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# Colon Cancer Specific Cytochrome P450 2W1: Polymorphism, Membrane Topology and Endogenous Roles in Development

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Cover page: the word cloud of the thesis text in the shape of colon cancer ribbon.

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# Colon Cancer Specific Cytochrome P450 2W1: Polymorphism, Membrane Topology and Endogenous Roles in Development

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*To my family*

# ABSTRACT

Cytochrome P450 2W1 (CYP2W1) belongs to a family of drug metabolizing monooxygenases with an unidentified yet endogenous function. CYP2W1 expression pattern has characteristic features of oncofetal genes. Previous studies have shown significant levels of CYP2W1 protein in 30% of human colon cancers and in 50% of corresponding liver metastases. High expression of CYP2W1 is an independent prognostic factor for poor survival outcomes in colorectal cancer (CRC) patients. Such tumor specific feature renders CYP2W1 an attractive target for colon cancer therapy. Indeed, CYP2W1-mediated activation of a number of duocarmycin related chloromethylindolines demonstrated strong and selective cytotoxic effects toward cancer cells *in vitro* and *in vivo*.

CYP2W1 is a polymorphic gene. The most frequent genetic variants, are *CYP2W1*2* and *CYP2W1*6* that carry missense mutations producing amino acid changes that might affect the enzyme function. A recent study suggested that *CYP2W1*2* variant is associated with decreased risk of colon cancer. We have analyzed a 10-fold larger patient cohort where no such differences in the distribution of *CYP2W1*2* and *CYP2W1*6* between CRC patients and healthy controls could be detected. Moreover, the functional analysis of these enzyme variants demonstrated equally potent capacity to metabolize the chloromethylindoline substrates.

CYP2W1 enzyme is glycosylated at Asn177 in cancer tissues and in transfected cells, suggesting that in contrast to other P450s it has an inverted endoplasmic reticulum (ER) membrane topology with the bulk of the protein facing the lumen. We now confirmed such membrane orientation based on a number of assays using FLAG-tagged CYP2W1 and also CYP2W1 fused with a redox sensitive luciferase reporter that displays full activity only upon its oxidative folding in the ER. Such ER topology apparently should hinder CYP2W1 interaction with its canonical cytosol oriented redox partners, cytochrome P450 reductase (POR) and/or cytochrome $b_5$ reductase. This was confirmed by unaffected functional activity of CYP2W1 under the conditions of knocked down/inhibited POR and cytochrome $b_5$ reductase. Interestingly, the nonglycosylated Asn177Ala mutant displayed significantly decreased catalytic activity. This study strongly supports the atypical membrane topology of CYP2W1 and suggests the interaction of CYP2W1 with a hypothetical ER luminal electron donor.

CYP2W1 is transiently expressed during gut development. We described a developmental expression profile of CYP2W1 in murine and human gastrointestinal tissues. The gene expression is initiated at the early stages of gestation and is completely silenced shortly after birth. Such fetal specific expression was shown to be epigenetically regulated and indicates possible endogenous role in gut development. Cyp2w1 knock out (KO) mouse model was established in order to investigate such possibility. Phenotype analysis revealed decreased crypt length in the distal colon epithelium of adult Cyp2w1 KO mice whereas the transcriptomic profiling identified a number of genes being down-regulated. The most affected genes were found to be involved in the cell adhesion and extracellular matrix establishment.

In conclusion, we have characterized polymorphic variants of CYP2W1 and found no significant correlation between their distribution and CRC risk. The completely inverted membrane topology of catalytically active CYP2W1 suggests its interaction with redox partner yet to be identified in the ER lumen. The explicitly developmental pattern of expression, the phenotypic and gene expression changes in Cyp2w1 KO gut tissues indicate a potential role of CYP2W1 in gut development.

# LIST OF PUBLICATIONS

This thesis is based on the following papers which are referred to in the text by their Roman numerals:

I. Kristina Stenstedt*, Sandra Travica*, **Jia Guo**, Isabel Barragan, Klaus Pors, Laurence Pattersson, David Edler, Souren Mkrtchian, Inger Johansson, Magnus Ingelman-Sundberg. CYP2W1 polymorphism: functional aspects and relation to risk for colorectal cancer. *Pharmacogenomics. 2013; 14(13): 1615-22.*

II. Eva Choong, **Jia Guo**, Anna Persson, Susanne Virding, Inger Johansson, Souren Mkrtchian, Magnus Ingelman-Sundberg. Developmental regulation and induction of cytochrome P450 2W1, an enzyme expressed in colon tumors. *PLoS One. 2015; 10(4): e0122820.*

III. **Jia Guo**, Stefanie Thiess, Inger Johansson, Souren Mkrtchian, Magnus Ingelman-Sundberg. Membrane topology and search for potential redox partners of colon cancer specific cytochrome P450 2W1. *FEBS Lett. 2016; 590(3):330-9.*

IV. **Jia Guo**, Marin Jukic, Inger Johansson, Souren Mkrtchian, Magnus Ingelman-Sundberg. The oncofetal enzyme CYP2W1: role in gut development. *Manuscript.*

Related review article:

**Jia Guo**, Inger Johansson, Souren Mkrtchian, Magnus Ingelman-Sundberg. The CYP2W1 enzyme. Regulation, properties and activation of prodrugs. *Drug Metabolism Reviews. 2016; accepted for publication.*

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 6PGDH | 6-phosphogluconate dehydrogenase |
| 11β-HSD1 | 11 beta hydroxysteroid dehydrogenase type 1 |
| ACN | Acetonitrile |
| Ala | Alanine |
| Asn | Asparagine |
| CEA | Carcinoembryonic antigen |
| CNV | Copy number variation |
| CRC | Colorectal cancer |
| CYB5/ Cyt b5 | Cytochrome $b_5$ |
| CYB5R | Cytochrome $b_5$ reductase |
| CYP/ P450 | Cytochrome P450 |
| DPI | Diphenyleneiodonium chloride |
| E | Embryonic day |
| ER | Endoplasmic reticulum |
| FFA | Free fatty acid |
| G6PDH | Glucose-6-phosphate dehydrogenase |
| Gluc | *Gaussia* luciferase |
| GG | Gluc-GFP tandem reporter |
| H6PD | Hexose-6-phosphate dehydrogenase |
| HCC | Hepatocellular carcinoma |
| H&E | Hematoxylin and eosin |
| HH | Hedgehog |
| HPLC | High performance liquid chromatography |
| IHC | Immunohistochemistry |
| IMM | Inner mitochondria membrane |
| Km | Michaelis constant, substrate concentration that gives half maximal velocity of an enzymatic reaction |
| KO | Knockout |
| LC-MS | Liquid chromatography–mass spectrometry |
| LPC | Lysophosphatidylcholines |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| PMT | Prodrug monotherapy |

| | |
|---|---|
| PND | Postnatal day |
| POR | Cytochrome P450 oxidoreductase |
| PPP | Pentose phosphate pathway |
| PTU | 6-Propyl-2-thiouracil |
| RA | Retinoic acid |
| SNP | Single nucleotide polymorphism |
| SRS | Substrate recognition site |
| Vmax | The maximal velocity of a reaction |
| WT | Wild type |

# 1 INTRODUCTION

## 1.1 Cytochrome P450 Superfamily

### 1.1.1 Function

The name of Cytochrome P450 (CYP, or P450) originates from several decades ago when Tsuneo Omura and Ryo Sato in 1962 described a hemoprotein nature of liver microsomal pigment as "P-450": it has a unique character of maximal spectral absorbance at 450 nm when the reduced form binds carbon monoxide (Omura and Sato, 1962). Since then the literature on cytochrome P450 research has expanded significantly. 57 CYP genes and 58 pseudogenes have been identified in the human genome (Nelson et al., 2004, Ingelman-Sundberg, 2005). The P450 superfamily is divided into families and subfamilies which are grouped by their primary structure similarity: the ones with > 40% amino acid sequence identity belong to one family, and the ones with > 55% identity are sorted into one subfamily (Nelson, 2006).

*Evolution*

Cytochrome P450s are ubiquitously expressed in various species spanning from *Bacteria*, *Archaea* to *Eukarya*. It is postulated that the first cytochrome P450 gene arose about 3.5 billion years ago when the oxygen-rich atmosphere was not yet in place, and acted as nitroreductase. Years of evolution expanded the CYP family which plays critical roles in the proper functioning of organisms. The first expansion gave rise to CYP11 and CYP4 families which are designated for metabolism of endogenous cholesterol, fatty acids that are essential to maintain the membrane integrity of early eukaryotic cells. Another expansion of the CYP family brought about CYP19, CYP21 and CYP27 families which are involved in the synthesis of endogenous steroids. Later expansions further enriched the CYP superfamily with CYP1-3 family members that are capable of metabolizing xenobiotics including environmental toxicants, procarcinogens and drugs, as well as endogenous substrates (Danielson, 2002, Lewis et al., 1998).

*Enzyme catalysis*

CYP is a superfamily of heme-containing monoxygenases that catalyze the introduction of one oxygen atom into the substrates, whereas the other oxygen atom is reduced to a water molecule: $RH + O_2 + 2H^+ + 2\ e^- \longrightarrow ROH + H_2O$ (Meunier et al., 2004).

In eukaryotic cells CYPs are anchored to the endoplasmic reticulum (ER) or inner mitochondrial membrane (IMM) by the N-terminal hydrophobic signal peptide, keeping the majority of enzyme including the bulk of catalytic domain and redox interactive domain in the cytosol (type III ER membrane protein, as described below in the membrane protein topology section) or mitochondrial lumen (Black, 1992). Such topological orientation facilitates electron transfer from their redox partners, cytochrome P450 reductase (POR), cytochrome $b_5$ in ER or adrenodoxin (Fdx1) in mitochondria (Porter, 2002, Ewen et al., 2011, Hannemann et al., 2007).

*Redox cofactor $NAD(P)^+/NAD(P)H$*

NADH and NADPH are the major electron carriers in P450 mediated catalytic cycle. They are produced mostly in the cytosol in various metabolic reactions. The cytosolic NADPH is generated during the pentose phosphate pathway (PPP) by glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) using $NADP^+$ as an electron acceptor (Lunt and Vander Heiden, 2011, Pollak et al., 2007). The cytosolic NADPH/$NADP^+$ pool is overall in reduced state, maintaining the antioxidant milieu. In addition, a fraction of NADPH/$NADP^+$ exists also in the ER lumen. Since the ER membrane is scarcely permeable to pyridine nucleotides, the luminal NADPH is produced separately by hexose-6-phosphate dehydrogenase (H6PD) and the 11 beta-hydroxysteroid dehydrogenase type 1 (11ß-HSD1) is one of the major NADPH consuming enzymes. These enzymes work in tight connection to sustain the luminal NADPH homeostasis (Csala et al., 2006, Banhegyi et al., 2004, Czegle et al., 2006, Banhegyi et al., 2012).

### 1.1.2 Structure and substrate binding

Mammalian CYPs can be divided into two groups according to their substrate binding specificity: one group with narrow substrate specificity, which is mostly involved in the biosynthesis, activation or inactivation of endogenous regulatory molecules; the other group shows very broad substrate binding spectrum, representing the xenobiotic

metabolizing P450s.

The three dimensional (3D) structure analysis of cytochrome P450 can provide the molecular perspective of substrate recognition and binding sites. The microbial P450s are fully soluble proteins while mammalian P450s are membrane bound. Despite only 20-30% of amino acid identity between prokaryotic and eukaryotic P450s, the overall fold was predicted to be conserved, which is why the structure-function studies of mammalian P450s were previously based on the bacterial P450 structural model (e.g. CYP101/ Cytochrome P450cam) (Li and Poulos, 1996, Schlichting et al., 1997, Li and Poulos, 1997, Podust et al., 2001). The obstacle for the determination of the crystal structure of mammalian P450s is the hydrophobic amino-terminal hydrophobic fragment. Therefore, in 2000, the first structure of membrane-associated P450, the rabbit CYP2C5 was solved by truncating of the N-terminal transmembrane helix and modulating the solubility of the second superficial membrane-binding region (Williams et al., 2000, Cosme and Johnson, 2000). Since then, the 3D structures of a few other mammalian P450s have been published including rabbit CYP2B4 and human CYP2C8, CYP2C9, CYP3A4, CYP2D6, CYP2A6 and CYP1A2, *etc.* (Scott et al., 2003, Williams et al., 2003, Schoch et al., 2004, Williams et al., 2004, Rowland et al., 2006, Yano et al., 2005, Sansen et al., 2007). The advancement of the X-ray crystallography of the substrate-bound proteins allowed the establishment of the substrate recognition site (SRS) map of CYPs, which made possible prediction of the substrate-binding residues. There are six putative SRS regions in eukaryotic P450s that are scattered along the primary structure and account for 16% of the total amino acid residues (Zawaira et al., 2011, Gotoh, 1992).

### 1.1.3 Extrahepatic P450s

CYPs are mostly expressed in the liver, and are responsible for the biotransformation of clinically administrated drugs, other xenobiotics (e.g. alcohol, procarcinogens) and endogenous compounds into easily excreted, hydrophilic metabolites. A number of CYPs is detected also in extrahepatic tissues and organs, including brain, heart, kidney, adrenal, thymus, *etc*., which could be important in regulating the metabolism of xenobiotics and/or endogenous substrates at the local sites (Karlgren et al., 2005). Many CYPs are found in respiratory and gastrointestinal (GI) tracts, the entry sites of the xenobiotics into the human body, e.g. lung, nasal tissue, small intestine, colon, *etc.*

A very detailed summary of the expression and regulation of extrahepatic CYPs in these tissues can be found in the review by Ding and Kaminsky, 2003 (Ding and Kaminsky, 2003).

Most of the CYPs identified in extrahepatic tissues are still mainly expressed in the liver, however, there are several CYPs that are preferentially expressed in extrahepatic tissues, which can lead to a tissue-specific metabolism profile of xenobiotics or endogenous substances. For instance, the CYP2S1 enzyme is highly expressed in human skin, small intestine, kidney with low expression level in the liver. Researchers found that there are significant individual variations in CYP2S1 cutaneous expression levels and it can be induced by ultraviolet radiation and *all-trans* retinoic acid. This implies that the topical drugs can be metabolized at different rates (Smith et al., 2003, Saarikoski et al., 2005)

The CYPs from family 1-3 that are expressed in gastrointestinal tract account for a part of the first-pass effect of orally administered drugs (Doherty and Charman, 2002). CYP3A4 is the most pronounced P450s in small intestine, other well documented GI CYPs include CYP1A1, CYP2C9, CYP2C19, CYP2J2, CYP2D6 (Paine et al., 2006, Kaminsky and Zhang, 2003). Clinical studies showed that a few drugs, e.g. midazolam, nifedipine, tacrolimus are mostly metabolized in the intestinal mucosa. Besides the CYP1-3 enzymes, there are also other CYPs in the GI tract, which are involved in the metabolism of endogenous substrates, such as CYP4 enzymes, CYP24A1 (1,25-dihydroxyvitamin D3 24-hydroxylase), CYP26A1, CYP27B1 (25 hydroxyvitamin D3-1-alpha hydroxylase) etc. (Kumarakulasingham et al., 2005, Anderson et al., 2006). Within the GI tissues, most CYPs are expressed in the intestinal epithelial cells, such as enterocytes or goblet cells, the very front line that interacts with xenobiotics. Other GI tissues also show CYPs expression: CYP1B1 is found in blood vessel walls and high levels of CYP2J2 are detected in autonomic ganglia, intestinal smooth muscle cells and vascular endothelium (Gibson et al., 2003, Zeldin et al., 1997).

### 1.1.4 P450 Polymorphism and precision medicine

On January 20, 2015 U.S. president Obama announced the Precision Medicine Initiative (PMI), ushering in a new era of individualized medical care that takes into account the interindividual variations in genes, environment and lifestyles for disease

prevention and treatment. Pharmacogenetics investigates polymorphic changes in the drug metabolizing genes causing interindividual variations in the drug response.

Cytochrome P450s are responsible for the Phase I metabolism (oxidation) of 70% clinically prescribed drugs directly determining the efficacy and turnover rate of the drugs by their inactivation or activation of the prodrugs. Despite of their importance, P450 genes exhibit high variability among individuals. The genetic variations include single nucleotide polymorphism (SNP), copy number variation (CNV), insertions and deletions. The nonsynonymous SNPs usually cause amino acid substitutions while CNV may alter gene expression level, which ultimately affect the enzyme catalytic activity and the pharmacokinetic property of relevant drugs.

The major polymorphic CYPs are from CYP2 family including CYP2A6, CYP2B6, CYP2C9, CYP2C19 and CYP2D6, as well as CYP3A5, which have been intensively studied and well characterized (Ingelman-Sundberg, 2004). Less has been reported on polymorphisms in CYP1A1, CYP1A2, CYP2C8, CYP2E1, CYP2J2, and CYP3A4. Polymorphisms in P450 redox partners such as cytochrome P450 reductase can also affect CYP enzyme function and catalytic activity (Hart and Zhong, 2008). The combinations of allelic variants result in different phenotypes which are known as poor, intermediate, extensive, and ultrarapid metabolizers (Ingelman-Sundberg et al., 2007).

Cancer chemotherapy is very unspecific and has narrow therapeutic window, which can cause severe or even fatal toxicity. However, lack of therapeutic effect due to excessive metabolism is also unwanted. Cytochrome P450s are responsible for the activation or inactivation of many anti-cancer drugs. One example is the highly polymorphic CYP2D6 mediating activation of tamoxifen, an estrogen receptor modulator used for prevention and treatment of breast cancer. Considering the high degree of genetic polymorphisms of these isoforms that can result in the altered catalytic activities and ultimately affect the drug efficacy, it’s imperative to perform genotyping for the prediction of catalytic activity of the enzyme and thus make possible the personalized treatment (Rodriguez-Antona and Ingelman-Sundberg, 2006, Ruddy et al., 2013)

## 1.2 Cytochrome P450 2W1

CYP2W1 is one of the latest members in cytochrome P450 superfamily, which is so far an orphan enzyme with undefined function. The first partial sequence of human *CYP2W1* gene was identified in a cDNA library from human hepatoma cell line HepG2 in 2000 and later the full length cDNA was cloned (Karlgren et al., 2006). The *CYP2W1* gene is evolutionarily conserved. The human *CYP2W1* gene is located on chromosome 7, consists of 9 exons, and has the highest sequence identity with other two CYP2 family members, *CYP2D6* and *CYP2S1*. A number of publications illuminated various aspects of this enzyme including its expression profile, specific substrates, regulation, polymorphisms and its potential role in colon cancer therapy.

### 1.2.1 Oncofetal expression

Among various extrahepatic CYP enzymes, CYP2W1 has shown a unique oncofetal expression pattern. Significant amounts of CYP2W1 transcript are identified in the fetal rat colon tissues (Karlgren et al., 2006), however the enzyme was not found in any adult tissue. Choudhary et al showed that Cyp2w1 is transcribed in pooled fetal mouse tissue (Choudhary et al., 2005). A detailed developmental curve of CYP2W1 expression in human and mouse colon and intestine is presented in paper II.
The scanning of the RNA panel of multiple human tumor tissues revealed that significant amount of CYP2W1 mRNA is present in human colon tumor tissues, and to a lesser extent in the adrenal gland tumors. However the CYP2W1 expression is absent in any of examined normal tissues using multiple tissue expression array and multiple tissue northern blot, indicating a tumor specific expression pattern (Karlgren et al., 2006). With the development of the CYP2W1 specific antibody, several studies provided with the solid evidence of high CYP2W1 protein expression in colon tumors (Gomez et al., 2007, Edler et al., 2009, Stenstedt et al., 2014, Stenstedt et al., 2012). In addition, CYP2W1 tumor expression profiles have been expanded by a flood of recent publications which show various levels of CYP2W1 expression in breast cancer (Bandala et al., 2012), childhood rhabdomyosarcoma (Molina-Ortiz et al., 2014), hepatocellular carcinoma (Zhang et al., 2014), adrenocortical carcinoma (Ronchi et al., 2014), oral squamous cell carcinoma (Hartanto et al., 2015). Due to the limited specificity of commercial CYP2W1 antibodies, further validations are needed for most of these cancer types. Moreover, high levels of the enzyme are also detected in several

cancer derived cell lines including the hepatoma cell line HepG2, colorectal carcinoma cell lines Caco2-TC7, HCC2998 and KM12, breast cancer cell line MCF7 (Tan et al., 2011).

### 1.2.2 Prognostic factor for CRC and potential role in targeted therapy

Two clinical studies have analyzed CYP2W1 expression in colorectal cancer patients and sought for potential correlation between the enzyme level and cancer malignancy. In the first cohort, 162 tumors from patients with stages II and III colorectal cancer (CRC) were analyzed. Among them, 64% of tumors displayed significant CYP2W1 levels as revealed by immunohistochemistry detection while the signal from the nascent normal tissues was negligible, and 36% showed very intensive staining. The high expression of CYP2W1 is associated with worse clinical outcome of overall survival rate, which is an independent prognostic factor in multivariate analysis ($p=0.04$) (Edler et al., 2009). Another independent cohort consisted of 235 malignant colon tumors from stage II and III CRC patients to exam CYP2W1 expression. In line with the previous study, high level of CYP2W1 was detected in 30% of the tumors. CYP2W1 expression is an independent prognostic factor in the patients with stage III colorectal cancer ($p=0.003$) (Stenstedt et al., 2012). A follow-up study on primary colorectal tumors and the corresponding lymph node and liver metastases revealed high CYP2W1 expression in 50% of metastatic tissues, which promises good perspectives for targeting CYP2W1 in metastases (Stenstedt et al., 2014). Recently, Zhang K, et al. suggested the prognostic value of CYP2W1 expression in hepatocellular carcinoma (HCC) which is based on a cohort study of 133 HCC patients (Zhang et al., 2014).

Tumor specific expression of CYP2W1 offers attractive opportunities for the development of targeted cancer therapy. A library of duocarmycin derivatives that can be converted into cytotoxic metabolites by CYP2W1 was developed at the University of Bradford, UK (Sheldrake et al., 2013). The inactive chloromethylindoline duocarmycin analogues undergo hydroxylation followed by spirocyclization to form the cyclopropane ring, thus becoming potent DNA-alkylating agents and being able to induce DNA replication stalling and cell death. Among them, ICT2705 and ICT2706 emerged to exhibit very high affinity towards CYP2W1 in the stably transfected cell lines and showed potent cytotoxicity while the mock cells without CYP2W1 expression remained intact. Further, ICT2706 was tested *in vivo* in a xenograft mouse model of colon cancer. As shown in Figure 1, the results demonstrated significant tumor growth

inhibitory effect of ICT2706 in a CYP2W1 dependent manner. Moreover, such cytotoxicity can be propagated by the bystander effect to the surrounding cells in close proximity through the transfer of the active metabolite. Pharmacokinetics analysis of tumor and plasma concentrations after the administration of ICT2706 showed the accumulation of the compound preferentially in tumor tissues. Taken together, these studies suggest a possibility of a novel colon cancer therapy based on the CYP2W1 bioactivation of anticancer prodrug at local sites (Travica et al., 2013). Moreover, a non-toxic chloromethylindoline CYP2W1 substrate ICT2726 was suggested as a probe to detect the enzyme activity prior to the therapy.

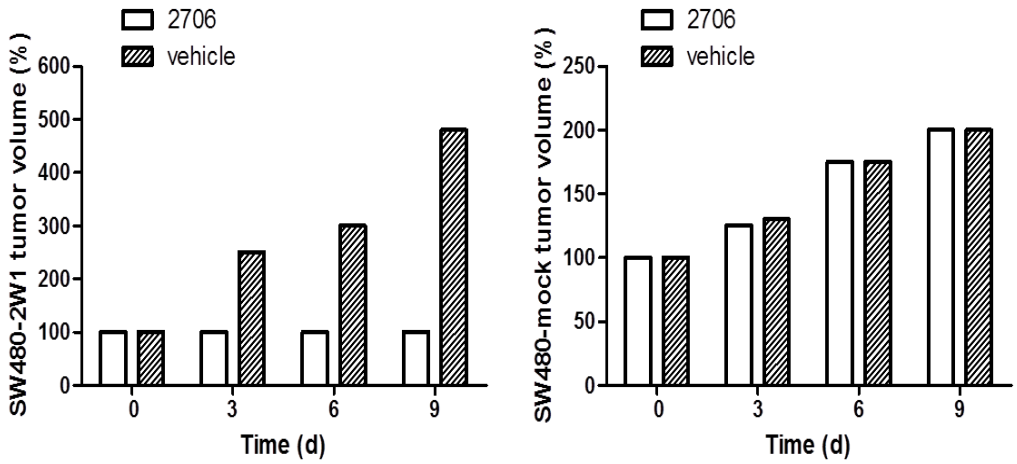



**Figure 1.** ICT2706 inhibits tumor growth in the mouse xenograft model of human colon cancer. Left: Tumors with CYP2W1 expression (SW480-2W1); right: Tumors without CYP2W1 expression (SW480-mock). Adapted from (Travica et al., 2013).

### 1.2.3 Substrate specificity

Although being grouped into the CYP2 family, CYP2W1 demonstrates the most similarity in substrate binding with the CYP1 family member, CYP1A1, which is in line with the substrate recognition site (SRS) prediction (Karlgren and Ingelman-Sundberg, 2007). Efforts have been made to demonstrate catalytic capability of CYP2W1 towards various substrates. Most studies were carried out in reconstituted systems with the *E. coli* expressed N-terminally truncated human CYP2W1 and NADPH or NADPH regenerating system (Mazur et al., 2009). The putative CYP2W1 substrates can be categorized briefly into three types: I. procarcinogens; II. anti-tumor drugs; III. endogenous compounds.

The reported procarcinogens include aromatic amines, polycyclic aromatic hydrocarbon dihydrodiols, sterigmatocystin, which have relatively low turnover rates by CYP2W1 (Wu et al., 2006, Eun et al., 2010). The anti-tumor drugs, such as AQ4N, GW610, 5F203 (Wang and Guengerich, 2012, Tan et al., 2011) have also shown to be substrates of CYP2W1. However, the 5F203 is predominantly metabolized by CYP1A1, whereas fluorobenzothiazole GW610 shows more specificity towards CYP2W1. Low metabolism rates have been shown in CYP2W1 mediated benzphetamine demethylation, and indole oxidation (Wu et al., 2006, Yoshioka et al., 2006). Meanwhile, a series of putative endogenous substrates was suggested for CYP2W1. A systematic study has been carried out by Xiao Y and Guengerich FP where the colorectal tumor extracts were incubated with purified recombinant CYP2W1 enzyme. The metabolites were analyzed by LC-MS. The results showed that CYP2W1 catalyzes selective hydroxylation or epoxidation of lysophospholipids, lysophosphatidylcholines (LPC) in particular and free fatty acids (FFA) (Xiao and Guengerich, 2012). Another recent study showed that CYP2W1 has tight binding constants for retinoids including *all-trans* retinol, *all-trans* retinal and *all-trans* retinoic acids at nanomolar level (Zhao et al., 2016b). This is, so far, the highest affinity reported for any CYP2W1 substrates. Considering the important signaling function of retinoids in cell proliferation, differentiation and carcinogenesis, it was suggested that CYP2W1 might play a role in tumor development via mediating retinoids metabolism. The study also reported less efficient metabolism towards 17 ß-estradiol, farnesol, arachidonic acid. However, these experiments were accomplished under anaerobic conditions, which necessitates confirmation *in vitro* (cell system) and *in vivo*.

Stable expression of CYP2W1 in mammalian cells provides a useful tool to explore its specific substrates under physiological situations. The CYP2W1 transfected HEK293 cells reveal catalytic activity towards indolines and aflatoxin $B_1$ (Gomez et al., 2010) which paved the road for later development of the chloromethylindoline duocarmycin analogues as described above. The nontoxic substrate ICT2726 has a Km value of 3.46±1.17 µM in the CYP2W1 transfected SW480 cells (Stenstedt et al., 2013).

### 1.2.4 Regulation

It is well established that the expression of many cytochrome P450 genes are regulated by nuclear receptors such as pregnane X receptor (PXR), constitutive androstane

receptor (CAR), aryl hydrocarbon receptor (AhR) on transcriptional level. However, the extrahepatic CYP2W1 has a unique oncofetal expression pattern, which suggests possible involvement of epigenetic regulation. Initial study has shown that the treatment by demethylation reagent AzaC triggers the re-expression of the enzyme in human hepatoma cell line B16A2 that previously had no detectable CYP2W1, implying the significant role of epigenetic modification (Karlgren et al., 2006). Indeed, a CpG island was identified in the exon1-intron 1 junction of *CYP2W1* gene. An elegant study has shown that the methylation level of this locus in *CYP2W1* gene reversely correlates with its expression when comparing colon tumor tissue to the corresponding normal tissue (Gomez et al., 2007). DNA methylation thus represents the primary, if not the only, mechanism that explains the tumor specific expression of CYP2W1. Studies presented in paper II further demonstrate the role of epigenetic modification in determining the transient fetal expression of CYP2W1 in both human and mice gut tissues. It is also shown that CYP2W1 expression could be induced by anti-tumor reagents e.g. GW610, imatinib in several carcinoma cell lines (Tan et al., 2011), (paper II), suggesting the involvement of undefined tissue specific transcriptional regulators. However, PXR agonist rifampicin and AhR agonist TCDD are unable to enhance CYP2W1 expression (presented in paper II) which exclude their participation in modulating CYP2W1 transcription.

### 1.2.5 Polymorphism

The members of CYP2 family e.g. CYP2C9, CYP2C19, CYP2D6 are known for their high frequency of polymorphism. Not surprisingly, several single nucleotide polymorphisms (SNP) have been described in *CYP2W1* gene too. A study covering all nine exons and exon-intron junctions on Japanese population reported six nonsynonymous SNPs which lie in the exon 1, 4 and 9. In haplotype analysis, the allelic variants are characterized (*CYP2W1*2-*6*) with *CYP2W1*1* as the reference (Hanzawa et al., 2008). Among them, *CYP2W1*6* displays the most significant frequency with amino acid substitution Pro488Leu. Ten novel SNPs have been described recently in Chinese Uygur and Han populations with seven SNPs in the exons and the other three in the introns. Four novel non-synonymous polymorphisms producing amino acid substitutions might affect CYP2W1 function. The allelic frequencies are different among Chinese Han, Uygur and Caucasians, e.g. 2.9%, 5.2%, 9.1% for *CYP2W1*2* and 0%, 0%, 33.1% for *CYP2W1*3* (Qi et al., 2015).

Considering the prognostic value of CYP2W1 in colon cancer, it was postulated that *CYP2W1* alleles might be associated with increased or decreased cancer risk. Gervasini G et al. investigated the frequency differences of nonsynonymous SNPs in *CYP2W1* gene between CRC patients and healthy subjects and the result suggested that *CYP2W1*2 (Ala181Thr)* is associated with lower risk for CRC (Gervasini et al., 2010). In Paper I, a thorough study based on a much larger cohort has been carried out in order to illuminate the relationship between CYP2W1 genotype and colon cancer risk as well as the catalytic function of the enzyme variants.

### 1.2.6 Posttranslational modification

Western blot detection of CYP2W1 in the transfected cells and cancer tissues consistently reveals two immunoreactive bands (52 and 54 kDa), indicating that CYP2W1 might be subject to posttranslational modification (Aguiar et al., 2005, Gomez et al., 2010). *In silico* analysis by the NetNGlyc software predicted possible *N*-glycosylation at Asn177 site. Site-directed mutation of Asn177 residue resulted in the disappearance of the upper band (54 kDa) which confirmed the glycosylation of CYP2W1 enzyme. Moreover, a migration shift from 54 kDa to 52 kDa of the CYP2W1 immunoreacitve bands was observed when the microsomal fractions from transfected cells or colon tumors were treated with deglycosylating enzymes, EndoH and PNGase (Gomez et al., 2010). Cytochrome P450s have typical cytosolic orientation, thus making them inaccessible to the ER luminal residing glycosylation machinery. In order to solve this puzzle, it is critical to determine the membrane topology of CYP2W1. Protease protection assay suggested that CYP2W1 may have an inverted topology with the majority of the molecule orienting towards ER lumen, thus enabling the glycosylation process. It was previously reported that a small fraction of CYP enzymes are inversely anchored to the ER membrane and can be further directed to the plasma membrane (Neve and Ingelman-Sundberg, 2010, Neve and Ingelman-Sundberg, 2000). Indeed, 8.1% of total CYP2W1 is localized on the cell surface of transfected HEK293 cells as detected by biotinylation assay (Gomez et al., 2010). However, given the fact that the canonical P450 reductases (POR, cytochrome $b_5$ reductase) are oriented toward cytosol, one would wonder how CYP2W1 obtains electrons having such an atypical orientation. Investigation regarding the membrane topology and search for potential redox partner of CYP2W1 is illustrated in paper III.

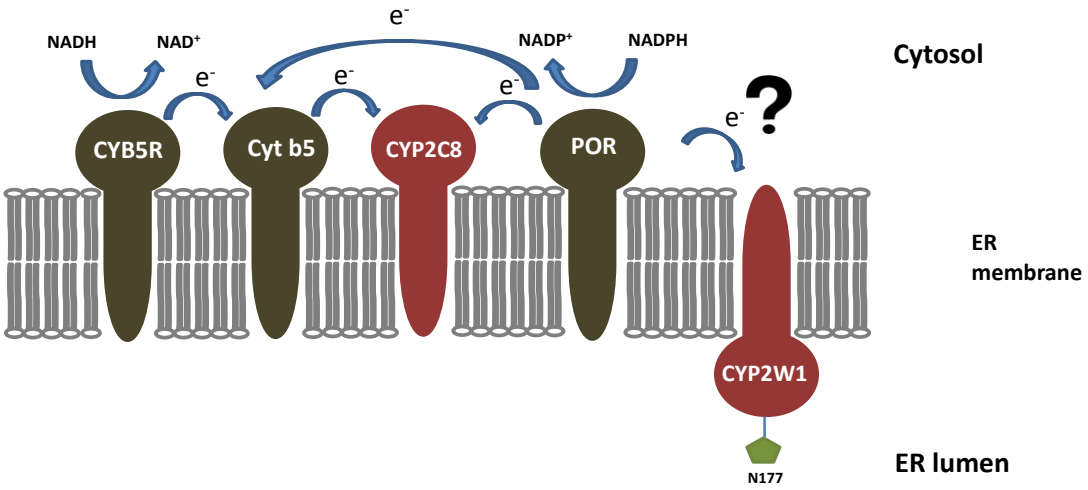



**Figure 2.** The electron transfer chain to typical CYPs (e.g. CYP2C8) and the posttranslational modification and atypical orientation of CYP2W1. CYB5R: cytochrome $b_5$ reductase; Cyt b5: cytochrome $b_5$; POR: P450 reductase.

## 1.3 Membrane Protein Topology

### 1.3.1 Membrane topology

The definition of membrane topology is "a specification of the number of transmembrane helices and their in and/or out orientations across the membrane in a membrane protein." (von Heijne, 2006). Topology has its great importance in determining the fully folded three dimensional (3D) structures of membrane proteins from the amino-acid sequence. With recent development in the field of X-ray crystallography, databases have expanded by exponentially increasing numbers of high-resolution 3D structures of membrane proteins, which enrich the concept of topology with deeper understanding and more complex interpretation. For instance, the membrane-embedded helices can be so various: they could cross the membrane by oblique angles or just lie flat on the membrane surface, or form the "re-entrant" loops by spanning only a part of membrane and turning back. Moreover, if one considers how to define the transmembrane helix, it's not clear how far exactly the helix should reach the interface regions on both sides of the membrane to be qualified (von Heijne, 2006). Nevertheless, we hereby discuss the single spanning membrane protein topology adopting the less complicated definition.

### 1.3.2 Protein translocation and targeting to endoplasmic reticulum

The proteins are translated from messenger RNAs on cytosolic ribosomes and are targeted to their corresponding subcellular locations. Generally, the incorporation of proteins into endoplasmic reticulum (ER) membrane are directed by certain signals which are characterized as uncharged, predominantly hydrophobic N-terminal sequences of 7-25 amino acids. The membrane proteins that are tethered to ER can be divided into three groups, according to their ways of inserting the single or multiple-spanning transmembrane helixes into the ER membrane. The type I membrane proteins initiate the translocation by their C-terminal sequence; the cleavable signal at N-terminus is cleaved off by signal peptidase and new luminal N'-terminus is generated; type II membrane proteins have similar translocation pattern at their C-terminus, however the N-terminus remains uncleaved and span the hydrophobic core of the bilayer with an $N_{cyt}/C_{lum}$ orientation (N-terminus in cytosol, C-terminus in ER lumen); type III or often classified as type Ia, induce the translocation of the N-terminus with reverse signal anchors, resulting in an $N_{lum}/C_{cyt}$ orientation (Higy et al.,

2004). A typical example of type III ER membrane protein is cytochrome P450 enzyme.

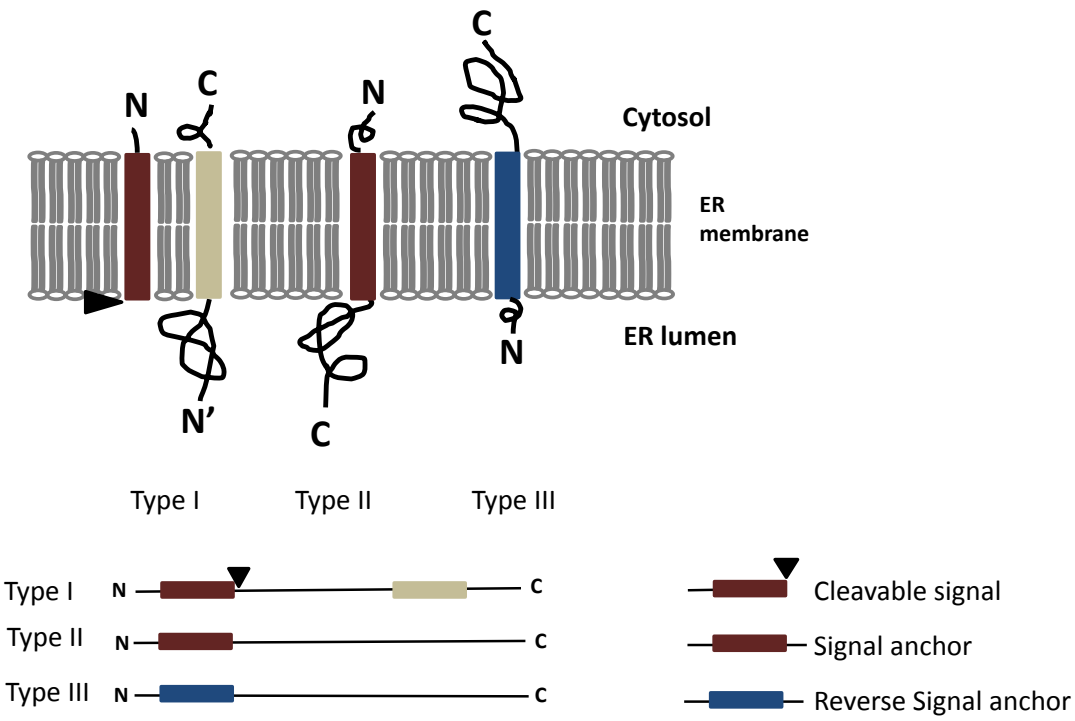



**Figure 3.** The three types of ER membrane protein, adapted from (Higy et al., 2004). Type I and type II proteins have the signal-anchor sequences (red) that induce the translocation of the C-terminus and assume an $N_{cyt}/C_{lum}$ orientation. The arrowhead indicates cleavable signal. Type III protein has reverse signal anchor (blue) that translocates the N-terminus and inserts with an opposite $N_{lum}/C_{cyt}$ orientation. Grey bar: additional transmembrane segment.

### 1.3.3 Methods for membrane topology determination

The primary role of the membrane in cells is to separate different organelles from the cellular matrix and ensure the individual compartments function well in their specific roles. For instance, endoplasmic reticulum, as the protein processing machinery for proper folding, employs the membrane to maintain a relatively oxidative environment in the lumen in order to facilitate the protein disulfide bond formation. In contrary, on the other side of the membrane, the cytoplasm has a rather reductive environment. Therefore, the orientation of the membrane protein and its specific topology underlies the correct functionality as it determines the accessibility of the protein to its cofactors or substrates within certain microenvironment.

Given the important role of topology for protein function, different approaches are developed in order to probe the topological distribution.

*Computational predictions.* A number of web-based *in silico* prediction approaches have been developed over the last decades (OCTOPUS, SCAMPI-single, SCAMPI-multi, TOPCONS, Tmpred, *etc.*), providing immediate and fundamental structural information of the transmembrane proteins, e.g. examination of the relative hydrophobicity of different peptide regions of a protein to predict membrane-spanning domains. Considering the difficulties in obtaining the structural knowledge experimentally for transmembrane proteins, computational methods are of exceptional significance. However, the prediction results of such methods are, as expected, not solid, and different prediction methods often give inconsistent outcomes that are based on different algorithms, leading to uncertain result (Bernsel et al., 2009). Neither can they provide detailed information regarding to whether the N- or C- terminus faces the cytosol, lumen or cell exterior.

*Engineering epitope* is another category of approaches for characterizing the topological distribution. Epitopes such as hemagglutinin (HA), FLAG and myc, are tethered to N- or C- terminus of the protein of interest. Immunoreactivity of the epitopes is further analyzed by antibody labelling before and after the permeabilization of cellular membrane. Such methods are applied mostly to distinguish between extracellular and intracellular orientation. Similar principle is adopted by protease protection assay to unravel the more detailed topological knowledge at the subcellular organelle level (Jarvik and Telmer, 1998).

*Fluorescence protease protection (FPP)* is a recently developed assay to determine the membrane protein topology in living cells (Lorenz et al., 2006). Briefly, a fluorescent protein is attached to the N- or C-terminus of the questioned protein. Cells expressing the fusion protein are treated with trypsin before or after the exposure to low concentration digitonin, which can efficiently permeabilize the plasma membrane while keeping the intracellular membranes intact. The plasma membrane proteins facing the cell exterior would be the very first to be digested by trypsin, as extracellular trypsin is inaccessible to the interior of intact cells. Meanwhile, the loss of fluorescence signal when digitonin is added indicates that the fluorescently tagged protein faces cytoplasm. Conversely, if the fluorescence signal is not affected when exposed to trypsin and digitonin, it indicates a luminal orientation of the protein of

interest in subcellular organelles, e.g. ER, Golgi apparatus, mitochondria or peroxisomes.

*Redox-sensitive luciferase assay* provides with a powerful tool to characterize the localization and topology of endoplasmic reticulum proteins. As mentioned above, the redox environment in the ER lumen is particularly oxidative compared to cytoplasm, which confers the redox sensitive reporter with alternating activity modes depending on specific orientation and topology of either single- or multiple-pass transmembrane proteins in ER. One of the redox sensitive proteins, *Gaussia* luciferase (Gluc) is isolated from the copepod marine organism *Gaussia princep*, consisting of 185 amino-acid. The proper folding of Gluc, which is possible only in the oxidative environment of ER lumen, enables the catalysis of coelenterazine oxidation, producing quantitative bioluminescent signal, which is > 10-fold higher than in cytosol. The tandem Gluc-GFP (GG), where GFP is used to normalize the protein expression level, is designed as a redox sensitive reporter that can be fused to different positions of a target protein to determine its topology (Li et al., 2012, Tsachaki et al., 2015).

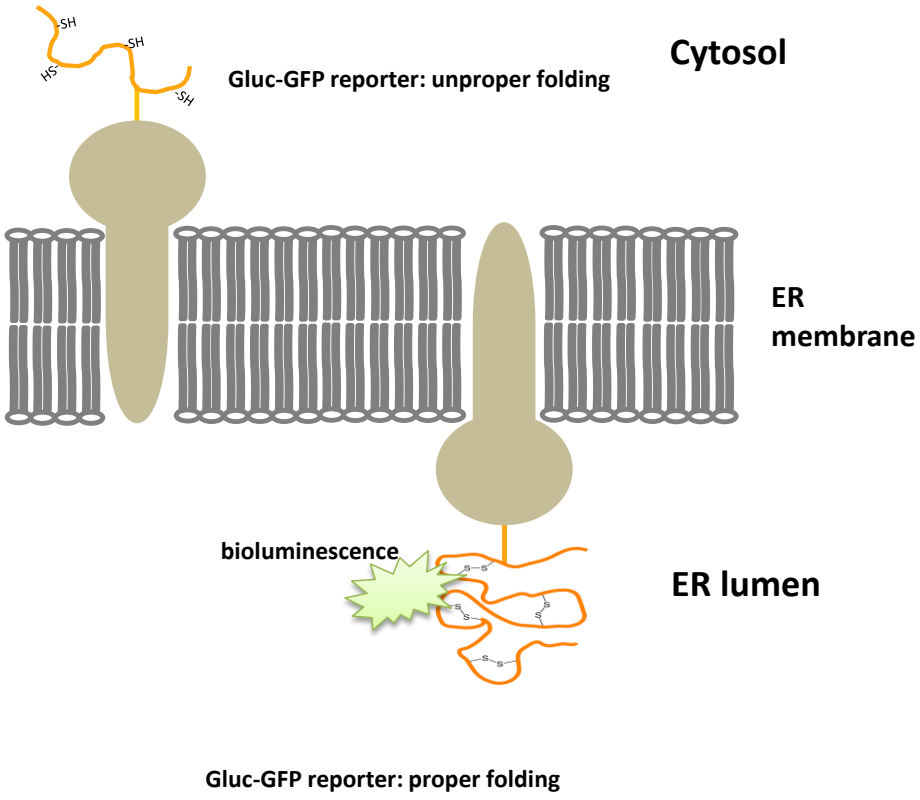



**Figure 4.** The tandem Gluc-GFP (GG) redox sensitive reporter. Oxidative folding of Gluc in the ER lumen produces an active enzyme.

## 1.4 Parallels between Embryogenesis and Carcinogenesis

Embryonic development is featured as a well-regulated process of programmed cell proliferation and differentiation in order to shape an organized and "well behaved" structure. On the contrary, cancer is a "community" that has the least governance, and is notorious for harboring enormous genetic or epigenetic abnormalities. Despite the apparent distinctions between embryogenesis and carcinogenesis, there are quite a few common cellular and molecular hallmarks between them (Aiello and Stanger, 2016).

### 1.4.1 Re-activation of developmental signaling pathways in cancer

Cancer is characterized by re-activation of various embryonic signals in adulthood which arise from accumulated genetic mutations and epigenetic abnormality. The critical signaling pathways in development including Wnt, Hedgehog (HH) and Notch, are frequently dysregulated to a large extent in cancer and are involved in the initiation, progression of primary tumor growth and distant metastases in different cancer types (Aiello and Stanger, 2016).

In colorectal cancer, the transcriptional recapitulation of a large cluster of embryonic genes is demonstrated by comparing the expression pattern of human CRC and mouse colon tumor models to those of the embryonic mouse colon during E13.5-18.5 (Kaiser et al., 2007). The cancer activated genes are functionally associated with cell cycle progression, proliferation, and migration. The identification of their normal counterparts' role in embryogenesis would likely provide valuable insights for cancer therapeutic strategies (Hu and Shivdasani, 2005, Hendrix et al., 2007).

### 1.4.2 Examples of oncofetal genes

*Carcinoembryonic antigen (CEA)* is a group of cell adhesion glycoproteins, belonging to the immunoglobulin superfamily. They were identified in 1965 from human colon cancer tissue extracts, and are very widely used as biomarkers for prognosis and prediction of recurrence after surgical resection. Regarding to ontogeny, CEA is produced in gastrointestinal tissues during fetal development, and the expression is silenced in adult tissue (Hammarstrom, 1999, Benchimol et al., 1989).

*SALL4 in hepatocellular carcinoma* The *SALL4* gene encodes a C2H2 zinc-finger transcription factor which is a critical regulator in maintaining the pluripotency and self-renewal of embryonic stem cells. The expression of *Sall4* in murine liver decreases continuously during the development and is undetectable in adult liver, indicating its potential role in fetal liver development during early-to-middle stages. *SALL4* gene is found to be re-expressed in various types of cancers including hepatocellular carcinoma, acute myeloid leukemia, lymphoma, breast cancer etc., and is essential for cancer cell proliferation, thus defining it as an oncogene (Kobayashi et al., 2011, Ma et al., 2006, Cui et al., 2006, Oikawa et al., 2013). Remarkably, the study on hepatocellular carcinoma patients reveals 55.6% positivity of SALL4 expression in tumor samples. Differential expression of SALL4 is observed in matched tissue samples with up-regulated levels in neoplastic specimens, and the high expression level of SALL4 in hepatocellular carcinoma patients is associated with poor prognostic outcome (Yong et al., 2013).

### 1.4.3 P450s in development

The fetal development of the mammals relies on fine-tuned spatio-temporal expression of regulators in various signaling pathways, some of which are synthetized or metabolized by CYP enzymes (Rifkind et al., 1995). There is growing evidence for the important functions of CYPs during embryogenesis. One example is that the complete depletion of cytochrome P450 reductase POR, the primary electron donor for microsomal P450s, in mouse leads to multiple developmental defects observed early at day 10.5 of gestation, and results in embryonic lethality by embryonic day 13.5 (E13.5) (Shen et al., 2002).

The first systematic investigation of mouse CYP expression patterns during development was done by Choudhary et al. The mRNAs of 40 known mouse *Cyp* genes were quantified from E7 until E17 (*Cyp2w1* gene was not included). A number of CYP members are constitutively expressed through all developmental stages, including Cyp2s1, Cyp8a1, Cyp20, Cyp21a1, Cyp26a1, Cyp46, and Cyp51 (Choudhary et al., 2003). CYP26 are the major enzymes that metabolize the *all-trans* retinoic acid (RA). Both the Cyp26a1 and Cyp26b1 null mice are lethal at neonatal stage (Abu-Abed et al., 2001, Yashiro et al., 2004). Cyp26 enzymes have important role in establishing an uneven distribution of RA along the anterio-posterior axis,

which is essential for the hindbrain patterning (Sakai et al., 2001). CYP2S1, CYP2W1 and CYP1B1 are also reported to metabolize retinoids (Saarikoski et al., 2005, Zhao et al., 2016a, Choudhary et al., 2004).

Cyp1b1 protein demonstrates a spatio-temporal expression pattern during mouse eye ontogeny and plays an important role in the normal eye development (Choudhary et al., 2007). Cyp1b1 knockout mice reveals developmental abnormalities of the drainage structures of the eye resembling those reported in human primary congenital glaucoma (PCG) (Libby et al., 2003). In human, CYP1B1 immunostaining shows more intense signal in fetal eyes when compared to adult eyes (Doshi et al., 2006). It is well documented that *CYP1B1* gene mutation is a common cause of human PCG, potential future clinical treatment is proposed for glaucoma patients by delivering the wild type *CYP1B1* gene to adult stem cells from eye (Choudhary et al., 2009, Vasiliou and Gonzalez, 2008).

# 2 AIMS

The overall aim of this thesis was to provide more insights into the genetic polymorphism, expression regulation, membrane topology and endogenous function of CYP2W1 enzyme.

The specific aims were:

I. Investigation of the association between the CYP2W1 polymorphism and the risk of colorectal cancer, evaluation of the catalytic characteristics of CYP2W1 variant proteins.

II. Elucidation of the regulatory mechanisms of the fetal expression of CYP2W1 and search for potential inducers.

III. Characterization of the membrane topology and search for the redox partners of CYP2W1.

IV. Investigation of the endogenous function of CYP2W1 in colon development by a Cyp2w1 knockout mouse model.

# 3 RESULTS AND DISCUSSION

## 3.1 CYP2W1 Polymorphism: Functional Aspects and Relation to Risk for Colorectal Cancer (Paper I)

In the CYP superfamily genetic polymorphisms are rather prevalent among different populations, and the polymorphic gene variants can code for the enzymes that have increased or decreased catalytic activity. In the *CYP2W1* gene, six single nucleotide polymorphisms (SNPs) have been described in a study on Japanese population, which are located in the protein coding area causing amino acid changes. The variant alleles are designated as *CYP2W1*2* to *CYP2W1*6*, whereas the reference allele is defined as *CYP2W1*1* (Hanzawa et al., 2008).

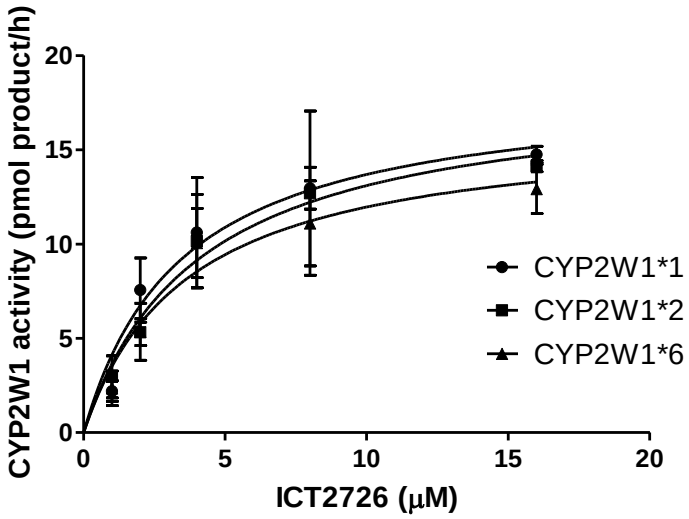
A previous study by Gervasini and coworkers suggested that *CYP2W1*2* genetic variant is associated with decreased risk of colorectal cancer based on clinical analysis and genotyping of genomic DNA samples from 150 colorectal cancer (CRC) patients and 263 healthy controls (Gervasini et al., 2010). In light of the availability of specific and high-affinity duocarmycin analogue substrates for CYP2W1 enzyme, it was of interest to study whether the genetic variants code for enzymes that display functional differences, and also validate their correlation with CRC risk on significantly larger cohort of patients.

In the current study 1785 CRC patients and 1761 healthy blood donors were included in order to analyze the distribution of three *CYP2W1* allelic variants (*CYP2W1*1*, *CYP2W1*2*, *CYP2W1*6*). The molecular epidemiological study revealed, in contrast to the previous study, that the allelic frequencies and genotypes of *CYP2W1* variants were equally distributed between the CRC patients and healthy controls. Therefore, our results do not support the decreased risk of CRC among *CYP2W1*2* carriers.

We further evaluated the functional aspects of the three variants. In transiently transfected colon cancer cells SW480 expressing CYP2W1.1, CYP2W1.2 and CYP2W1.6, the expression of each variant was determined to be at the same level by western blot. Duocarmycin analogues were used to investigate the catalytic activity of the CYP2W1 variants in the transfected cells. The duocarmycin prodrug ICT2706 was

incubated with transfected SW480 cells followed by cell viability assay, showing that there was no statistically significant difference between the three CYP2W1 enzyme variants in mediating the conversion of ICT2706 into cytotoxic products. Similar results were obtained when incubating cells with various concentrations of nontoxic substrate ICT2726. The enzyme kinetics results as presented in Figure 5 indicate that CYP2W1.2 and CYP2W1.6 have comparable catalytic activity as CYP2W1.1 with the equal substrate turnover efficiency.

Taken together, our study clarified that there is no significant difference in the distribution of three *CYP2W1* genetic variants between CRC patients and healthy controls. The functional analysis of CYP2W1.1, CYP2W1.2, and CYP2W1.6 revealed equal capability in metabolizing duocarmycin analogue substrates. Our study indicates that there is no prerequisite of genotyping CYP2W1 when applying the CYP2W1-targeted precision medicine to CRC patients.




| | CYP2W1*1 | CYP2W1*2 | CYP2W1*6 |
|---|---|---|---|
| $V_{max}$ | 18.44±2.24 | 18.44±1.80 | 16.33±2.06 |
| $K_m$ | 3.46±1.17 | 4.07±1.04 | 3.64±1.25 |

**Figure 5.** The enzyme kinetics of CYP2W1 variants mediated ICT2726 metabolism.

## 3.2 CYP2W1 Membrane Topology and Search for Electron Partners (Paper III)

As discussed in Introduction, cytochrome P450s are heme-containing monooxygenases which catalyze the oxidative reaction by incorporating one oxygen atom into substrates while the other oxygen atom is reduced to water. Such process is enabled by the electron transport chain in the cytosol, which consists of NADPH and cytochrome P450 reductase (POR), and in some cases NADH, cytochrome $b_5$ reductase and cytochrome $b_5$. The P450s tether to the membrane of endoplasmic reticulum with the N-terminal signal anchor peptide, while the major bulk of heme-containing domain faces the cytosol (Figure 2).

Previous studies have detected CYP2W1 enzyme by immunoblotting in colon cancer tissues and transfected HEK293 cells, demonstrating two separated immunoreactive bands, which implies the possibility of post-translational modification on CYP2W1 protein. Further investigation identified the extra upper immunoreactive band as the consequence of glycosylation at the amino acid residue Asn177 of the enzyme (Gomez et al., 2010). The treatment by deglycosylating enzymes along with the membrane solubilizing reagent results in the depletion of the upper band in microsomes from transfected HEK293 cells, indicating that CYP2W1 enzyme is mostly likely embedded in the lumen of ER adopting atypical membrane topology, which is in line with the luminal residing glycosylation machinery. However, such luminal orientation is counterintuitive to the nature of monooxygenases which requires interaction with the redox partners residing on the cytosolic side of the ER membrane. CYP2W1 has indeed shown very potent catalytic activity towards a series of duocarmycin analogues both *in vitro* and *in vivo* (Travica et al., 2013). In order to address this fundamental question, there are two possible hypotheses: I. CYP2W1 is not completely luminally oriented, and partially remains in the ordinary membrane topology facing cytosol, which explains the functional activity; II. CYP2W1 is completely inverted but there is an alternative reducing partner equivalent to POR or cytochrome $b_5$ in the ER lumen that contributes electrons to CYP2W1.

With regard to the first hypothesis, we have applied various experimental approaches including the traditional protease protection assay and a novel redox-sensitive luciferase assay to investigate CYP2W1's membrane topology. As mentioned

previously, the protease protection assay cannot determine whether the whole pool of CYP2W1 has inverted topology or not. Therefore, we introduced a luciferase reporter assay approach based on the redox sensitive luciferase (Gluc) from *Gaussia princeps* and a tandem green fluorescence protein (GFP) that are fused to the C-terminus of CYP2W1. The rationale is that Gluc luciferase requires the oxidative environment in the ER lumen to fold properly, emitting the luminescence signal at least ten folds higher than in cytosol, thus allowing to discriminate between the luminal and cytosolic localization of the protein in question (Li et al., 2012). The GFP is used to normalize the expression level of the fusion protein. The CYP2W1 fusion construct (2W1-GG) as well as the cytosolic (GG-cyto) and luminal (GG-ER) controls together with the CYP2C8 recombinant construct (2C8-GG) were transfected to HEK293 cells and analyzed in parallel. The result revealed that the tested constructs fall into two distinct groups according to the ratios of luminescence/fluorescence signals: the constructs 2W1-GG and GG-ER belonged to one group that has very high bioluminescence signal compared to the other group consisting of 2C8-GG and GG-cyto. These data confirmed the canonical cytosolic topology of CYP2C8 and moreover indicated the completely luminal orientation of CYP2W1. The protease protection assay verified such topology by showing the luminal residence of the C-terminal of CYP2W1 utilizing a C-terminus FLAG tagged CYP2W1 construct.

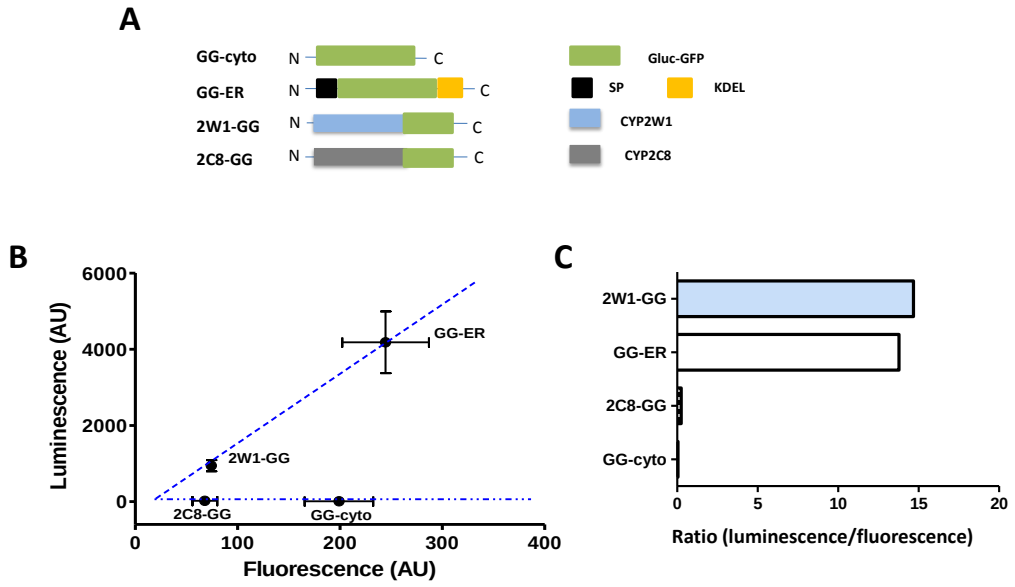



**Figure 6.** The redox sensitive luciferase reporter assay indicates the completely luminal orientation of CYP2W1. SP: signal peptide; KDEL: C-terminal ER-retention signal.

The inverted topology of CYP2W1 challenges the postulate of the electron transport chain of P450s that involves redox partners POR and cytochrome $b_5$ facing cytosol. We

investigated whether POR is the electron donor of CYP2W1 by inhibiting the expression of POR using siRNA in stably transfected HEK293 cells. The results shown in Figure 7 clearly demonstrated the efficient knockdown of POR. Under such circumstances, the CYP2W1 in intact HEK293 cells maintained its robust catalytic activity towards specific substrate ICT2726 while CYP2C8 enzyme catalysis was substantially affected by the absence of POR. Identical results were found when DPI, an efficient POR inhibitor (McGuire et al., 1998), was added to the system. These data solidly proved that POR is not interacting with CYP2W1. Similar scenario was seen when PTU was employed to inhibit flavin reductases including cytochrome $b_5$ reductase (Lee and Kariya, 1986). Taken together these data indicate that CYP2W1 catalytic activity is not dependent on the presence of either POR or cytochrome $b_5$ reductase, suggesting other redox partner(s) in the lumen that may interact with CYP2W1.

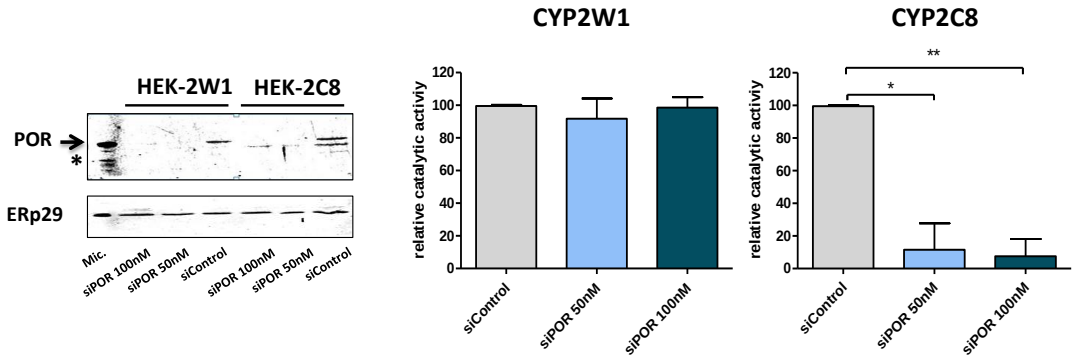



**Figure 7.** P450 reductase (POR) is not the electron donor to CYP2W1.

Interestingly, glycosylation has been found to be relevant for catalytic activity of CYP2W1 enzyme. The Asn177Ala (N177A) nonglycosylated mutant construct described previously (Gomez et al., 2010) was expressed in HEK293 cells and immunoblotting confirmed the complete absence of the glycosylation fraction. Catalytic activity characterization of the nonglycosylated mutant towards ICT2726 revealed significantly reduced metabolite formation when compared to the corresponding wild type CYP2W1 construct.

In summary, our findings strongly support the completely inverted topology of CYP2W1 facing ER lumen, and exclude canonical redox partners as electron suppliers to CYP2W1. This suggests the existence of the hypothetical luminal electron donor(s) that remain to be identified.

## 3.3 CYP2W1 in the Development

### 3.3.1 Developmental expression of CYP2W1 (Paper II)

The *CYP2W1* gene is evolutionary conserved with transcripts reported in rat fetal colon and in mouse embryonic pooled tissues (Karlgren et al., 2006, Choudhary et al., 2005), whereas expression is silenced in adult life. Previous study has elucidated that epigenetic modification underlines the tumor-specific expression of CYP2W1, in which the demethylation of the CpG island located in the exon1/intron 1 junction of *CYP2W1* gene results in activated transcription in colorectal tumors (Gomez et al., 2007). The expression pattern of CYP2W1 is reminiscent of a group of oncofetal genes, which are regulated by similar mechanisms during fetal development and carcinogenesis.

Previous reports of CYP2W1 expression in rat or murine fetal tissue were limited to the transcriptional level and the human fetal tissue expression data was not available. In the current study we analyzed the Cyp2w1 mRNA and protein expression levels in murine colon and small intestine tissues at several time points ranging from embryonic days 13 (E13) until adulthood. As shown in Figure 8. Cyp2w1 reached significant expression levels during fetal stages both in colon and small intestine with the peak level at E18 and then decreased drastically after birth until becoming undetectable at four weeks of age. However, there were slight differences in expression curves between the small intestine and colon: Cyp2w1 is expressed earlier in fetal small intestine and also diminished earlier at around postnatal day three (PND 3) while Cyp2w1 mRNA in colon tended to be more stable until PND 7. Western blot confirmed the protein expression of Cyp2w1 that shared the same pattern as mRNA in colon and small intestine. It is worth to mention that liver samples from different developmental stages were analyzed as well and we did not detect any Cyp2w1 transcripts at any time point.

Regarding to the CYP2W1 expression in human fetal colon and small intestine, samples from gestational weeks 18-19 were analyzed along with a few adult samples at various ages. As we expected, significant CYP2W1 expression was found in colon and small intestine during fetal stages and not detected at any adult ages, which is consistent with the murine data.

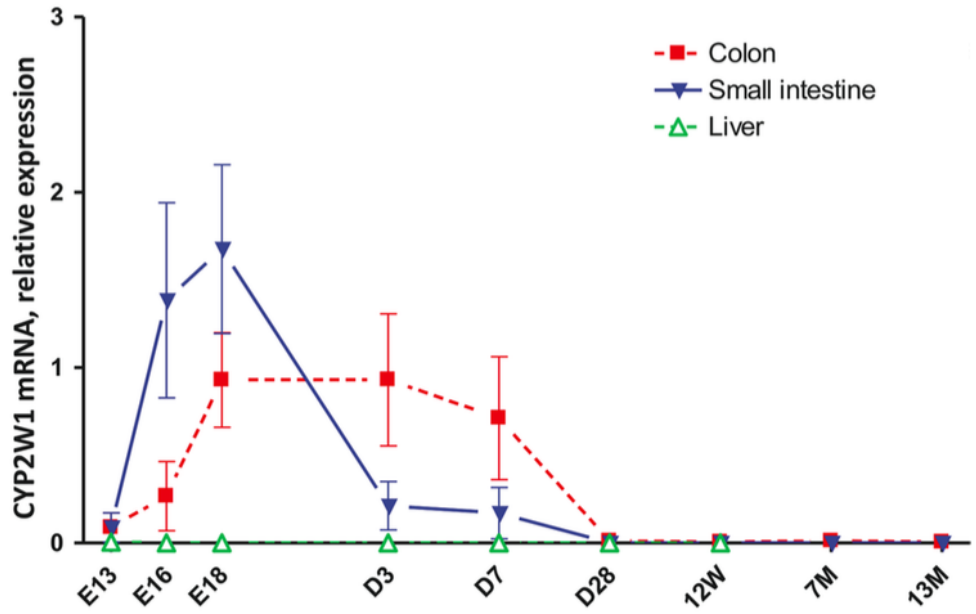



**Figure 8.** The developmental curve of Cyp2w1 mRNA expression in murine small intestine and colon.

It was also of interest to identify the cell types in fetal gut expressing CYP2W1. In the current study, immunohistochemical analysis using two CYP2W1 antibodies was carried out on the human fetal colon at gestational week 19. CYP2W1 signals were observed exclusively in the crypt cells lining the epithelium layer, co-localizing with the ER marker, GRP78, while the other layers including lamina propria, muscularis mucosa or submucosa have no CYP2W1 staining, as shown in Figure 9.

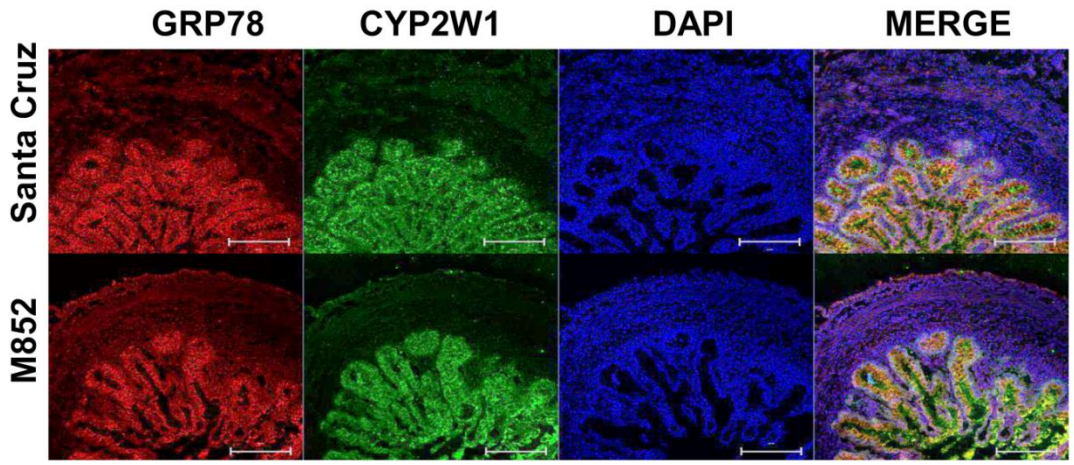



**Figure 9.** CYP2W1 expression in human fetal colon at gestational week 19. M852: human CYP2W1 in-house antibody; GRP78: endoplasmic reticulum marker.

### 3.3.2 Regulation of CYP2W1 (Paper II)

As reported previously, DNA demethylation of CpG island in exon 1/intron 1 junction triggers the expression of CYP2W1 in colon tumor tissue. We tested whether such mechanism is involved in the regulation of CYP2W1 expression in fetal gut. The methylation level of the exon 1/intron 1 region of human *CYP2W1* gene was compared between the fetal and postnatal colon tissue. The results revealed that six CpG sites within this region have higher methylation level in adult samples than in fetal tissues indicating the involvement of epigenetics in regulating the developmentally specific expression of CYP2W1, which is in consistent with the findings in colon cancers. The methylation states in murine pre- and postnatal colon samples showed significant correlation between Cyp2w1 mRNA expression and DNA methylation percentage in six analyzed areas of high CpG density sites.

CYP2W1 has significant expression levels in colon cancer but is not detectable in normal tissues, rendering it an attractive target for colon cancer therapy utilizing the prodrug monotherapy (PMT) concept (de Groot et al., 2001, Denny, 2004). When applying the PMT to colon cancer, it is beneficial to up-regulate CYP2W1 expression to obtain more significant outcome. In this study, we were able to identify a colon cancer cell line HCC2998 that endogenously expresses considerable amount of catalytically active CYP2W1. Utilizing the HCC2998 cell model for screening the potential inducers of CYP2W1, we found significant inducing effect on CYP2W1 mRNA levels when the cells were treated with imatinib, linoleic acid and its two conjugated forms. Imatinib is a tyrosine-kinase inhibitor used in the treatment of multiple cancers (Druker et al., 2006, van Oosterom et al., 2001), and its potent effect on CYP2W1 expression might open up a possibility of a neoadjuvant colon cancer therapy prior to the administration of prodrugs specifically activated by CYP2W1 to cytotoxins.

Collectively, in this study we have described a detailed developmental curve of CYP2W1 expression in mice and human gut tissues, revealed that the fetal specific expression is largely modulated by epigenetic regulation and found potential transcriptional inducers of CYP2W1.

### 3.3.3 Endogenous role of CYP2W1 in gut development (Paper IV, manuscript)

Since the first identification of a partial sequence from HepG2 cDNA library in 2000, several aspects of CYP2W1 have been investigated including substrate specificity, genetic polymorphism, epigenetic regulation, and most importantly, the tumor-specific expression, which promotes it as a promising target for the treatment of colon primary tumors and hepatic metastases. Despite the promising role of CYP2W1 in the context of colon cancer therapy, little is known however regarding its endogenous function, leaving it so far in the ranks of orphan enzymes.

Previously characterized expression pattern of CYP2W1 shares common features with the expression of oncofetal genes. The *CYP2W1* gene is evolutionarily conserved among various vertebrate species, and has very confined expression in the epithelium of the fetal gastrointestinal tract. It was postulated that CYP2W1 may have potential role during gut development.

In order to understand the endogenous function of CYP2W1, a mouse model was established with the genomic depletion of the enzyme. qPCR analysis of Cyp2w1 mRNA from fetal colon and small intestine validated the successful abolishment of the gene in such knockout mice. The gastrointestinal phenotype of the adult Cyp2w1 null mouse was investigated in comparison with the corresponding wild type littermates. As shown in Figure 10, we found that the crypt length of the distal colon in KO mice is significantly smaller than that in the WT mice, which appeared as a thinner epithelium layer by H&E staining. Such a phenotype was not shown in any other parts of the GI tract including proximal colon, duodenum, jejunum, and ileum.

Next, we aimed to explore the molecular mechanisms that are associated with such phenotypic alteration. The transcript profiling of the distal colon from adult WT and KO mice was accomplished by microarray analysis and over hundred genes were found to be differentially expressed. The pathway analysis identified that a number of down-regulated genes in KO mice colon were involved in the cell adhesion and extracellular matrix establishment. The genes with the most significantly reduced expression were selected including Myl9, Fn1, Flna, and Itga5 for qPCR validation. Apart from the adult colon samples, fetal colon tissues at E13 and E18 were included as well. The

mRNA levels of analyzed genes were significantly decreased in adult Cyp2w1 null mice distal colon compared to WT, confirming thus the transcriptomics data. With respect to the fetal expression levels of these four genes, there was no difference at E13, however, a decreasing expression tendency was observed at E18 in KO mice colon samples. The results indicated that the depletion of the fetal Cyp2w1 expression has an impact on the adult expression of these genes, however, the mechanisms are yet to be explored. Recent studies demonstrated CYP2W1's capability of catalyzing a variety of endogenous lipid molecules, in particular very high affinity were reported towards the retinoic acid (Zhao et al., 2016b, Xiao and Guengerich, 2012), which might be important in regulating the expression of the downstream genes in the gut tissues.

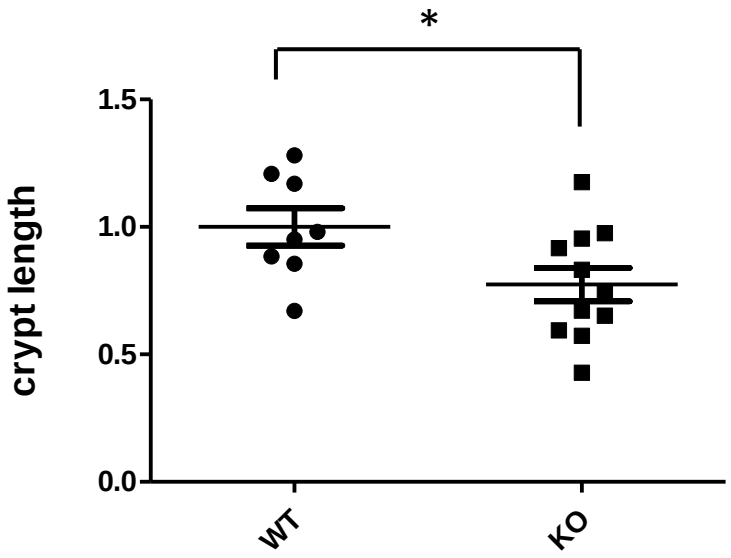



**Figure 10.** The crypt length of distal colon is significantly decreased in three months old Cyp2w1 knockout mice (KO) as compared to the wild type littermates (WT).

In summary, we have discovered the phenomenon of the decreased crypt length and revealed down-regulation of a number of cell adhesion genes in the Cyp2w1 KO mouse, providing thus more insights into a hypothetic endogenous function of CYP2W1 in the gut development.

# 4 SUMMARY

This thesis can be summarized as follows:

- The polymorphic *CYP2W1* gene variants are not related to the risk for colorectal cancer, which is in line with their equal functionality in metabolizing the specific substrates including anti-cancer prodrugs.

- CYP2W1 enzyme has a completely inverted membrane topology as compared to other P450s. Neither P450 reductase nor cytochrome $b_5$ reductase are involved in the electron transfer to CYP2W1. The nonglycosylated mutant has reduced catalytic activity compared to the wild type enzyme.

- CYP2W1 is expressed in the course of mouse and human embryonic gut development, and is silenced shortly after birth by DNA methylation. The enzyme localization is restricted to the crypt cells. Antitumor agent imatinib can induce CYP2W1 expression in colon carcinoma cell line HCC2998.

- The depletion of Cyp2w1 gene expression leads to the decreased crypt length of distal colon in adult mice, and is correlated with down regulation of a number of genes involved in cell adhesion.

# 5 FUTURE PERSPECTIVES

The establishment of the full sequence map of the human genome strongly contributed to the discoveries of many new genes including cytochrome P450 2W1. The initial characterization revealed tumor specific expression profile of the enzyme, which immediately caught our attention and suggested a novel cancer treatment approach by targeting CYP2W1 using the prodrug strategy. The success in synthesis of CYP2W1 specific substrates duocarmycin analogues and their promising tumor-inhibitory effect in *in vivo* colon tumor xenografts pushes this strategy further towards the clinical setting. The equal capacity of different CYP2W1 polymorphic variants in prodrug catalysis circumvents the need of screening the *CYP2W1* genotype in CRC patients. Elevated CYP2W1 expression is found in colon cancer metastases, our ongoing studies are dedicated to the analysis of the functional activity of CYP2W1 in the metastatic tissues and generation of a novel xenograft mouse model using liver metastatic tissue from CRC patients, in order to estimate the tumor-killing efficacy of duocarmycin prodrugs. In addition, we plan to study the mechanisms of metastatic dissemination investigating the effects of co-culture of a panel of colon cancer cells with the 3D spheroids of primary human hepatocytes.

The oncofetal expression pattern of CYP2W1 is governed by epigenetic mechanisms. DNA methylation of CYP2W1 gene leads to the expression silencing in adult tissues. The significant expression of CYP2W1 enzyme in course of the fetal gut development implies the potential endogenous role in embryogenesis. Indeed, the Cyp2w1 knockout mice revealed a phenotype of shortened crypt length in distal colon at adulthood, along with decreased expression of a number of cell adhesion proteins. Interestingly, this was not observed during fetal life when the translation of *Cyp2w1* gene occurs, although a tendency of down-regulation in cell adhesion genes was noticed. One possible explanation would be the long-lasting alterations in DNA methylation of the regulatory genes. Therefore, it would be interesting to investigate the epigenetic profiling of those down-regulated genes to characterize the regulatory elements or signaling pathways which might be associated with fetal CYP2W1 expression.

The recently reported high affinity binding constants of retinoids toward CYP2W1 implicates the potential involvement of the enzyme both in fetal development and in cancer progression. Retinoids are critical in regulating cell proliferation, differentiation, neuron development in the early embryogenesis. Moreover, retinoid signaling is shown to be undermined during tumor progression and retinoids are used in cancer therapy for proliferation arrest and differentiation induction (Tang and Gudas, 2011). Members of the CYP26 family are the major enzymes that metabolize retinoids into the less bioactive products. High levels of CYP26A1 are observed in several cancer types (Mongan and Gudas, 2007). With respect to the important role of retinoids in development and carcinogenesis, it is imperative to validate CYP2W1 mediated retinoid catalysis both in embryonic and in tumor tissues.

CYP2W1 has inverted membrane topology with the functional domain orienting toward ER lumen, which disables the redox interaction with canonical cytosol-facing CYP reductases. However, robust catalytic activity towards duocarmycin analogues has been shown in intact cells and in mice xenografts, indicating the existence of an ER luminal redox partner. Further investigations should identify the direct electron donor by means of RNA interfering or chemical inhibitors of luminal reductases, thus providing the additional opportunity of enhancing CYP2W1 catalytic activity via manipulation of its redox partner in cancer tissues.

In summary, the oncofetal CYP2W1 gene exhibits a temporal and spatial expression pattern and unique membrane topology. Future research may provide us with a clearer picture of CYP2W1 physiological function as well as with novel approaches for targeted cancer therapy.

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# REFERENCES


ABU-ABED, S., DOLLE, P., METZGER, D., BECKETT, B., CHAMBON, P. & PETKOVICH, M. 2001. The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. *Genes Dev,* 15**,** 226-40.

AGUIAR, M., MASSE, R. & GIBBS, B. F. 2005. Regulation of cytochrome P450 by posttranslational modification. *Drug Metab Rev,* 37**,** 379-404.

AIELLO, N. M. & STANGER, B. Z. 2016. Echoes of the embryo: using the developmental biology toolkit to study cancer. *Dis Model Mech,* 9**,** 105-14.

ANDERSON, M. G., NAKANE, M., RUAN, X., KROEGER, P. E. & WU-WONG, J. R. 2006. Expression of VDR and CYP24A1 mRNA in human tumors. *Cancer Chemother Pharmacol,* 57**,** 234-40.

BANDALA, C., FLORIANO-SANCHEZ, E., CARDENAS-RODRIGUEZ, N., LOPEZ-CRUZ, J. & LARA-PADILLA, E. 2012. RNA expression of cytochrome P450 in Mexican women with breast cancer. *Asian Pac J Cancer Prev,* 13**,** 2647-53.

BANHEGYI, G., BENEDETTI, A., FULCERI, R. & SENESI, S. 2004. Cooperativity between 11beta-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase in the lumen of the endoplasmic reticulum. *J Biol Chem,* 279**,** 27017-21.

BANHEGYI, G., MARGITTAI, E., SZARKA, A., MANDL, J. & CSALA, M. 2012. Crosstalk and barriers between the electron carriers of the endoplasmic reticulum. *Antioxid Redox Signal,* 16**,** 772-80.

BENCHIMOL, S., FUKS, A., JOTHY, S., BEAUCHEMIN, N., SHIROTA, K. & STANNERS, C. P. 1989. Carcinoembryonic antigen, a human tumor marker, functions as an intercellular adhesion molecule. *Cell,* 57**,** 327-34.

BERNSEL, A., VIKLUND, H., HENNERDAL, A. & ELOFSSON, A. 2009. TOPCONS: consensus prediction of membrane protein topology. *Nucleic Acids Res,* 37**,** W465-8.

BLACK, S. D. 1992. Membrane topology of the mammalian P450 cytochromes. *FASEB J,* 6**,** 680-5.

CHOUDHARY, D., JANSSON, I., REZAUL, K., HAN, D. K., SARFARAZI, M. & SCHENKMAN, J. B. 2007. Cyp1b1 protein in the mouse eye during development: an immunohistochemical study. *Drug Metab Dispos,* 35**,** 987-94.

CHOUDHARY, D., JANSSON, I. & SCHENKMAN, J. B. 2009. CYP1B1, a developmental gene with a potential role in glaucoma therapy. *Xenobiotica,* 39**,** 606-15.

CHOUDHARY, D., JANSSON, I., SCHENKMAN, J. B., SARFARAZI, M. & STOILOV, I. 2003. Comparative expression profiling of 40 mouse cytochrome P450 genes in embryonic and adult tissues. *Arch Biochem Biophys,* 414**,** 91-100.

CHOUDHARY, D., JANSSON, I., STOILOV, I., SARFARAZI, M. & SCHENKMAN, J. B. 2004. Metabolism of retinoids and arachidonic acid by human and mouse cytochrome P450 1b1. *Drug Metab Dispos,* 32**,** 840-7.

CHOUDHARY, D., JANSSON, I., STOILOV, I., SARFARAZI, M. & SCHENKMAN, J. B. 2005. Expression patterns of mouse and human CYP orthologs (families 1-4) during development and in different adult tissues. *Arch Biochem Biophys,* 436**,** 50-61.

COSME, J. & JOHNSON, E. F. 2000. Engineering microsomal cytochrome P450 2C5 to be a soluble, monomeric enzyme. Mutations that alter aggregation, phospholipid dependence of catalysis, and membrane binding. *J Biol Chem,* 275**,** 2545-53.

CSALA, M., BANHEGYI, G. & BENEDETTI, A. 2006. Endoplasmic reticulum: a metabolic compartment. *FEBS Lett,* 580**,** 2160-5.

CUI, W., KONG, N. R., MA, Y., AMIN, H. M., LAI, R. & CHAI, L. 2006. Differential expression of the novel oncogene, SALL4, in lymphoma, plasma cell myeloma, and acute lymphoblastic leukemia. *Mod Pathol,* 19**,** 1585-92.

CZEGLE, I., PICCIRELLA, S., SENESI, S., CSALA, M., MANDL, J., BANHEGYI, G., FULCERI, R. & BENEDETTI, A. 2006. Cooperativity between 11beta-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase is based on a common pyridine nucleotide pool in the lumen of the endoplasmic reticulum. *Mol Cell Endocrinol,* 248**,** 24-5.

DANIELSON, P. B. 2002. The cytochrome P450 superfamily: biochemistry, evolution and drug metabolism in humans. *Curr Drug Metab,* 3**,** 561-97.

DE GROOT, F. M., DAMEN, E. W. & SCHEEREN, H. W. 2001. Anticancer prodrugs for application in monotherapy: targeting hypoxia, tumor-associated enzymes, and receptors. *Curr Med Chem,* 8**,** 1093-122.

DENNY, W. A. 2004. Tumor-activated prodrugs--a new approach to cancer therapy. *Cancer Invest,* 22**,** 604-19.

DING, X. & KAMINSKY, L. S. 2003. Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu Rev Pharmacol Toxicol,* 43**,** 149-73.

DOHERTY, M. M. & CHARMAN, W. N. 2002. The mucosa of the small intestine: how clinically relevant as an organ of drug metabolism? *Clin Pharmacokinet,* 41**,** 235-53.

DOSHI, M., MARCUS, C., BEJJANI, B. A. & EDWARD, D. P. 2006. Immunolocalization of CYP1B1 in normal, human, fetal and adult eyes. *Exp Eye Res,* 82**,** 24-32.

DRUKER, B. J., GUILHOT, F., O'BRIEN, S. G., GATHMANN, I., KANTARJIAN, H., GATTERMANN, N., DEININGER, M. W., SILVER, R. T., GOLDMAN, J. M., STONE, R. M., CERVANTES, F., HOCHHAUS, A., POWELL, B. L., GABRILOVE, J. L., ROUSSELOT, P., REIFFERS, J., CORNELISSEN, J. J., HUGHES, T., AGIS, H., FISCHER, T., VERHOEF, G., SHEPHERD, J., SAGLIO, G., GRATWOHL, A., NIELSEN, J. L., RADICH, J. P., SIMONSSON, B., TAYLOR, K., BACCARANI, M., SO, C., LETVAK, L., LARSON, R. A. & INVESTIGATORS, I. 2006. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med,* 355**,** 2408-17.

EDLER, D., STENSTEDT, K., OHRLING, K., HALLSTROM, M., KARLGREN, M., INGELMAN-SUNDBERG, M. & RAGNHAMMAR, P. 2009. The expression of the novel CYP2W1 enzyme is an independent prognostic factor in colorectal cancer - a pilot study. *Eur J Cancer,* 45**,** 705-12.

EUN, C. Y., HAN, S., LIM, Y. R., PARK, H. G., HAN, J. S., CHO, K. S., CHUN, Y. J. & KIM, D. 2010. Bioactivation of Aromatic Amines by Human CYP2W1, An Orphan Cytochrome P450 Enzyme. *Toxicol Res,* 26**,** 171-5.

EWEN, K. M., KLESER, M. & BERNHARDT, R. 2011. Adrenodoxin: the archetype of vertebrate-type [2Fe-2S] cluster ferredoxins. *Biochim Biophys Acta,* 1814**,** 111-25.

GERVASINI, G., DE MURILLO, S. G., LADERO, J. M. & AGUNDEZ, J. A. 2010. CYP2W1 variant alleles in Caucasians and association of the CYP2W1 G541A (Ala181Thr) polymorphism with increased colorectal cancer risk. *Pharmacogenomics,* 11**,** 919-25.

GIBSON, P., GILL, J. H., KHAN, P. A., SEARGENT, J. M., MARTIN, S. W., BATMAN, P. A., GRIFFITH, J., BRADLEY, C., DOUBLE, J. A., BIBBY, M. C. & LOADMAN, P. M. 2003. Cytochrome P450 1B1 (CYP1B1) is overexpressed in human colon adenocarcinomas relative to normal colon: implications for drug development. *Mol Cancer Ther,* 2**,** 527-34.

GOMEZ, A., KARLGREN, M., EDLER, D., BERNAL, M. L., MKRTCHIAN, S. & INGELMAN-SUNDBERG, M. 2007. Expression of CYP2W1 in colon tumors: regulation by gene methylation. *Pharmacogenomics,* 8**,** 1315-25.

GOMEZ, A., NEKVINDOVA, J., TRAVICA, S., LEE, M. Y., JOHANSSON, I., EDLER, D., MKRTCHIAN, S. & INGELMAN-SUNDBERG, M. 2010. Colorectal cancer-specific cytochrome P450 2W1: intracellular localization, glycosylation, and catalytic activity. *Mol Pharmacol,* 78**,** 1004-11.

GOTOH, O. 1992. Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. *J Biol Chem,* 267**,** 83-90.

HAMMARSTROM, S. 1999. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. *Semin Cancer Biol,* 9**,** 67-81.

HANNEMANN, F., BICHET, A., EWEN, K. M. & BERNHARDT, R. 2007. Cytochrome P450 systems--biological variations of electron transport chains. *Biochim Biophys Acta,* 1770**,** 330-44.

HANZAWA, Y., SASAKI, T., MIZUGAKI, M., ISHIKAWA, M. & HIRATSUKA, M. 2008. Genetic polymorphisms and haplotype structures of the human CYP2W1 gene in a Japanese population. *Drug Metab Dispos,* 36**,** 349-52.

HART, S. N. & ZHONG, X. B. 2008. P450 oxidoreductase: genetic polymorphisms and implications for drug metabolism and toxicity. *Expert Opin Drug Metab Toxicol,* 4**,** 439-52.

HARTANTO, F. K., KAREN-NG, L. P., VINCENT-CHONG, V. K., ISMAIL, S. M., MUSTAFA, W. M., ABRAHAM, M. T., TAY, K. K. & ZAIN, R. B. 2015. KRT13, FAIM2 and CYP2W1 mRNA expression in oral squamous cell carcinoma patients with risk habits. *Asian Pac J Cancer Prev,* 16**,** 953-8.

HENDRIX, M. J., SEFTOR, E. A., SEFTOR, R. E., KASEMEIER-KULESA, J., KULESA, P. M. & POSTOVIT, L. M. 2007. Reprogramming metastatic tumour cells with embryonic microenvironments. *Nat Rev Cancer,* 7**,** 246-55.

HIGY, M., JUNNE, T. & SPIESS, M. 2004. Topogenesis of membrane proteins at the endoplasmic reticulum. *Biochemistry,* 43**,** 12716-22.

HU, M. & SHIVDASANI, R. A. 2005. Overlapping gene expression in fetal mouse intestine development and human colorectal cancer. *Cancer Res,* 65**,** 8715-22.

INGELMAN-SUNDBERG, M. 2004. Pharmacogenetics of cytochrome P450 and its applications in drug therapy: the past, present and future. *Trends Pharmacol Sci,* 25**,** 193-200.

INGELMAN-SUNDBERG, M. 2005. The human genome project and novel aspects of cytochrome P450 research. *Toxicol Appl Pharmacol,* 207**,** 52-6.

INGELMAN-SUNDBERG, M., SIM, S. C., GOMEZ, A. & RODRIGUEZ-ANTONA, C. 2007. Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoepigenetic and clinical aspects. *Pharmacol Ther,* 116**,** 496-526.

JARVIK, J. W. & TELMER, C. A. 1998. Epitope tagging. *Annu Rev Genet,* 32**,** 601-18.

KAISER, S., PARK, Y. K., FRANKLIN, J. L., HALBERG, R. B., YU, M., JESSEN, W. J., FREUDENBERG, J., CHEN, X., HAIGIS, K., JEGGA, A. G., KONG, S., SAKTHIVEL, B., XU, H., REICHLING, T., AZHAR, M., BOIVIN, G. P., ROBERTS, R. B., BISSAHOYO, A. C., GONZALES, F., BLOOM, G. C., ESCHRICH, S., CARTER, S. L., ARONOW, J. E., KLEIMEYER, J., KLEIMEYER, M., RAMASWAMY, V., SETTLE, S. H., BOONE, B., LEVY, S., GRAFF, J. M., DOETSCHMAN, T., GRODEN, J., DOVE, W. F., THREADGILL, D. W., YEATMAN, T. J., COFFEY, R. J., JR. & ARONOW, B. J. 2007. Transcriptional recapitulation and subversion of embryonic colon development by mouse colon tumor models and human colon cancer. *Genome Biol,* 8**,** R131.

KAMINSKY, L. S. & ZHANG, Q. Y. 2003. The small intestine as a xenobiotic-metabolizing organ. *Drug Metab Dispos,* 31**,** 1520-5.

KARLGREN, M., GOMEZ, A., STARK, K., SVARD, J., RODRIGUEZ-ANTONA, C., OLIW, E., BERNAL, M. L., RAMON Y CAJAL, S., JOHANSSON, I. & INGELMAN-SUNDBERG, M. 2006. Tumor-specific expression of the novel cytochrome P450 enzyme, CYP2W1. *Biochem Biophys Res Commun,* 341**,** 451-8.

KARLGREN, M. & INGELMAN-SUNDBERG, M. 2007. Tumour-specific expression of CYP2W1: its potential as a drug target in cancer therapy. *Expert Opin Ther Targets,* 11**,** 61-7.

KARLGREN, M., MIURA, S. & INGELMAN-SUNDBERG, M. 2005. Novel extrahepatic cytochrome P450s. *Toxicol Appl Pharmacol,* 207**,** 57-61.

KOBAYASHI, D., KURIBAYSHI, K., TANAKA, M. & WATANABE, N. 2011. SALL4 is essential for cancer cell proliferation and is overexpressed at early clinical stages in breast cancer. *Int J Oncol,* 38**,** 933-9.

KUMARAKULASINGHAM, M., ROONEY, P. H., DUNDAS, S. R., TELFER, C., MELVIN, W. T., CURRAN, S. & MURRAY, G. I. 2005. Cytochrome p450 profile of colorectal cancer: identification of markers of prognosis. *Clin Cancer Res,* 11**,** 3758-65.

LEE, E. & KARIYA, K. 1986. Propylthiouracil, a selective inhibitor of NADH-cytochrome b5 reductase. *FEBS Lett,* 209**,** 49-51.

LEWIS, D. F., WATSON, E. & LAKE, B. G. 1998. Evolution of the cytochrome P450 superfamily: sequence alignments and pharmacogenetics. *Mutat Res,* 410**,** 245-70.

LI, H. & POULOS, T. L. 1996. Conformational dynamics in cytochrome P450-substrate interactions. *Biochimie,* 78**,** 695-9.

LI, H. & POULOS, T. L. 1997. The structure of the cytochrome p450BM-3 haem domain complexed with the fatty acid substrate, palmitoleic acid. *Nat Struct Biol,* 4**,** 140-6.

LI, H. Y., ZHENG, X. M., CHE, M. X. & HU, H. Y. 2012. A redox-sensitive luciferase assay for determining the localization and topology of endoplasmic reticulum proteins. *PLoS One,* 7**,** e35628.

LIBBY, R. T., SMITH, R. S., SAVINOVA, O. V., ZABALETA, A., MARTIN, J. E., GONZALEZ, F. J. & JOHN, S. W. 2003. Modification of ocular defects in mouse developmental glaucoma models by tyrosinase. *Science,* 299**,** 1578-81.

LORENZ, H., HAILEY, D. W. & LIPPINCOTT-SCHWARTZ, J. 2006. Fluorescence protease protection of GFP chimeras to reveal protein topology and subcellular localization. *Nat Methods,* 3**,** 205-10.

LUNT, S. Y. & VANDER HEIDEN, M. G. 2011. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol,* 27**,** 441-64.

MA, Y., CUI, W., YANG, J., QU, J., DI, C., AMIN, H. M., LAI, R., RITZ, J., KRAUSE, D. S. & CHAI, L. 2006. SALL4, a novel oncogene, is constitutively expressed in human acute myeloid leukemia (AML) and induces AML in transgenic mice. *Blood,* 108**,** 2726-35.

MAZUR, C. S., KENNEKE, J. F., GOLDSMITH, M. R. & BROWN, C. 2009. Contrasting influence of NADPH and a NADPH-regenerating system on the metabolism of carbonyl-containing compounds in hepatic microsomes. *Drug Metab Dispos,* 37**,** 1801-5.

MCGUIRE, J. J., ANDERSON, D. J., MCDONALD, B. J., NARAYANASAMI, R. & BENNETT, B. M. 1998. Inhibition of NADPH-cytochrome P450 reductase and glyceryl trinitrate biotransformation by diphenyleneiodonium sulfate. *Biochem Pharmacol,* 56**,** 881-93.

MEUNIER, B., DE VISSER, S. P. & SHAIK, S. 2004. Mechanism of oxidation reactions catalyzed by cytochrome p450 enzymes. *Chem Rev,* 104**,** 3947-80.

MOLINA-ORTIZ, D., CAMACHO-CARRANZA, R., GONZALEZ-ZAMORA, J. F., SHALKOW-KALINCOVSTEIN, J., CARDENAS-CARDOS, R., NOSTI-PALACIOS, R. & VENCES-MEJIA, A. 2014. Differential expression of cytochrome P450 enzymes in normal and tumor tissues from childhood rhabdomyosarcoma. *PLoS One,* 9**,** e93261.

MONGAN, N. P. & GUDAS, L. J. 2007. Diverse actions of retinoid receptors in cancer prevention and treatment. *Differentiation,* 75**,** 853-70.

NELSON, D. R. 2006. Cytochrome P450 nomenclature, 2004. *Methods Mol Biol,* 320**,** 1-10.

NELSON, D. R., ZELDIN, D. C., HOFFMAN, S. M., MALTAIS, L. J., WAIN, H. M. & NEBERT, D. W. 2004. Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics,* 14**,** 1-18.

NEVE, E. P. & INGELMAN-SUNDBERG, M. 2000. Molecular basis for the transport of cytochrome P450 2E1 to the plasma membrane. *J Biol Chem,* 275**,** 17130-5.

NEVE, E. P. & INGELMAN-SUNDBERG, M. 2010. Cytochrome P450 proteins: retention and distribution from the endoplasmic reticulum. *Curr Opin Drug Discov Devel,* 13**,** 78-85.

OIKAWA, T., KAMIYA, A., ZENIYA, M., CHIKADA, H., HYUCK, A. D., YAMAZAKI, Y., WAUTHIER, E., TAJIRI, H., MILLER, L. D., WANG, X. W., REID, L. M. & NAKAUCHI, H. 2013. Sal-like protein 4 (SALL4), a stem cell biomarker in liver cancers. *Hepatology,* 57**,** 1469-83.

OMURA, T. & SATO, R. 1962. A new cytochrome in liver microsomes. *J Biol Chem,* 237**,** 1375-6.

PAINE, M. F., HART, H. L., LUDINGTON, S. S., HAINING, R. L., RETTIE, A. E. & ZELDIN, D. C. 2006. The human intestinal cytochrome P450 "pie". *Drug Metab Dispos,* 34**,** 880-6.

PODUST, L. M., POULOS, T. L. & WATERMAN, M. R. 2001. Crystal structure of cytochrome P450 14alpha -sterol demethylase (CYP51) from Mycobacterium tuberculosis in complex with azole inhibitors. *Proc Natl Acad Sci U S A,* 98**,** 3068-73.

POLLAK, N., DOLLE, C. & ZIEGLER, M. 2007. The power to reduce: pyridine nucleotides--small molecules with a multitude of functions. *Biochem J,* 402**,** 205-18.

PORTER, T. D. 2002. The roles of cytochrome b5 in cytochrome P450 reactions. *J Biochem Mol Toxicol,* 16**,** 311-6.
QI, G. Z., WANG, X., MIAO, X. J., YIN, S. J., REN, H., LOU, Y. Q. & ZHANG, G. L. 2015. Novel single nucleotide polymorphisms (SNPs) of CYP2W1 gene in Chinese Uygur and Han populations. *Drug Metab Pharmacokinet,* 30**,** 449-52.
RIFKIND, A. B., LEE, C., CHANG, T. K. & WAXMAN, D. J. 1995. Arachidonic acid metabolism by human cytochrome P450s 2C8, 2C9, 2E1, and 1A2: regioselective oxygenation and evidence for a role for CYP2C enzymes in arachidonic acid epoxygenation in human liver microsomes. *Arch Biochem Biophys,* 320**,** 380-9.
RODRIGUEZ-ANTONA, C. & INGELMAN-SUNDBERG, M. 2006. Cytochrome P450 pharmacogenetics and cancer. *Oncogene,* 25**,** 1679-91.
RONCHI, C. L., SBIERA, S., VOLANTE, M., STEINHAUER, S., SCOTT-WILD, V., ALTIERI, B., KROISS, M., BALA, M., PAPOTTI, M., DEUTSCHBEIN, T., TERZOLO, M., FASSNACHT, M. & ALLOLIO, B. 2014. CYP2W1 is highly expressed in adrenal glands and is positively associated with the response to mitotane in adrenocortical carcinoma. *PLoS One,* 9**,** e105855.
ROWLAND, P., BLANEY, F. E., SMYTH, M. G., JONES, J. J., LEYDON, V. R., OXBROW, A. K., LEWIS, C. J., TENNANT, M. G., MODI, S., EGGLESTON, D. S., CHENERY, R. J. & BRIDGES, A. M. 2006. Crystal structure of human cytochrome P450 2D6. *J Biol Chem,* 281**,** 7614-22.
RUDDY, K. J., DESANTIS, S. D., GELMAN, R. S., WU, A. H., PUNGLIA, R. S., MAYER, E. L., TOLANEY, S. M., WINER, E. P., PARTRIDGE, A. H. & BURSTEIN, H. J. 2013. Personalized medicine in breast cancer: tamoxifen, endoxifen, and CYP2D6 in clinical practice. *Breast Cancer Res Treat,* 141**,** 421-7.
SAARIKOSKI, S. T., RIVERA, S. P., HANKINSON, O. & HUSGAFVEL-PURSIAINEN, K. 2005. CYP2S1: a short review. *Toxicol Appl Pharmacol,* 207**,** 62-9.
SAKAI, Y., MENO, C., FUJII, H., NISHINO, J., SHIRATORI, H., SAIJOH, Y., ROSSANT, J. & HAMADA, H. 2001. The retinoic acid-inactivating enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the anterio-posterior axis within the mouse embryo. *Genes Dev,* 15**,** 213-25.
SANSEN, S., YANO, J. K., REYNALD, R. L., SCHOCH, G. A., GRIFFIN, K. J., STOUT, C. D. & JOHNSON, E. F. 2007. Adaptations for the oxidation of polycyclic aromatic hydrocarbons exhibited by the structure of human P450 1A2. *J Biol Chem,* 282**,** 14348-55.
SCHLICHTING, I., JUNG, C. & SCHULZE, H. 1997. Crystal structure of cytochrome P-450cam complexed with the (1S)-camphor enantiomer. *FEBS Lett,* 415**,** 253-7.
SCHOCH, G. A., YANO, J. K., WESTER, M. R., GRIFFIN, K. J., STOUT, C. D. & JOHNSON, E. F. 2004. Structure of human microsomal cytochrome P450 2C8. Evidence for a peripheral fatty acid binding site. *J Biol Chem,* 279**,** 9497-503.
SCOTT, E. E., HE, Y. A., WESTER, M. R., WHITE, M. A., CHIN, C. C., HALPERT, J. R., JOHNSON, E. F. & STOUT, C. D. 2003. An open conformation of mammalian cytochrome P450 2B4 at 1.6-A resolution. *Proc Natl Acad Sci U S A,* 100**,** 13196-201.
SHELDRAKE, H. M., TRAVICA, S., JOHANSSON, I., LOADMAN, P. M., SUTHERLAND, M., ELSALEM, L., ILLINGWORTH, N., CRESSWELL, A. J., REUILLON, T., SHNYDER, S. D., MKRTCHIAN, S., SEARCEY, M., INGELMAN-SUNDBERG, M., PATTERSON, L. H. & PORS, K. 2013. Re-engineering of the duocarmycin structural architecture enables bioprecursor

development targeting CYP1A1 and CYP2W1 for biological activity. *J Med Chem,* 56**,** 6273-7.
SHEN, A. L., O'LEARY, K. A. & KASPER, C. B. 2002. Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH-cytochrome P450 oxidoreductase. *J Biol Chem,* 277**,** 6536-41.
SMITH, G., WOLF, C. R., DEENI, Y. Y., DAWE, R. S., EVANS, A. T., COMRIE, M. M., FERGUSON, J. & IBBOTSON, S. H. 2003. Cutaneous expression of cytochrome P450 CYP2S1: individuality in regulation by therapeutic agents for psoriasis and other skin diseases. *Lancet,* 361**,** 1336-43.
STENSTEDT, K., HALLSTROM, M., JOHANSSON, I., INGELMAN-SUNDBERG, M., RAGNHAMMAR, P. & EDLER, D. 2012. The expression of CYP2W1: a prognostic marker in colon cancer. *Anticancer Res,* 32**,** 3869-74.
STENSTEDT, K., HALLSTROM, M., LEDEL, F., RAGNHAMMAR, P., INGELMAN-SUNDBERG, M., JOHANSSON, I. & EDLER, D. 2014. The expression of CYP2W1 in colorectal primary tumors, corresponding lymph node metastases and liver metastases. *Acta Oncol,* 53**,** 885-91.
STENSTEDT, K., TRAVICA, S., GUO, J., BARRAGAN, I., PORS, K., PATTERSON, L., EDLER, D., MKRTCHIAN, S., JOHANSSON, I. & INGELMAN-SUNDBERG, M. 2013. CYP2W1 polymorphism: functional aspects and relation to risk for colorectal cancer. *Pharmacogenomics,* 14**,** 1615-22.
TAN, B. S., TIONG, K. H., MURUHADAS, A., RANDHAWA, N., CHOO, H. L., BRADSHAW, T. D., STEVENS, M. F. & LEONG, C. O. 2011. CYP2S1 and CYP2W1 mediate 2-(3,4-dimethoxyphenyl)-5-fluorobenzothiazole (GW-610, NSC 721648) sensitivity in breast and colorectal cancer cells. *Mol Cancer Ther,* 10**,** 1982-92.
TANG, X. H. & GUDAS, L. J. 2011. Retinoids, retinoic acid receptors, and cancer. *Annu Rev Pathol,* 6**,** 345-64.
TRAVICA, S., PORS, K., LOADMAN, P. M., SHNYDER, S. D., JOHANSSON, I., ALANDAS, M. N., SHELDRAKE, H. M., MKRTCHIAN, S., PATTERSON, L. H. & INGELMAN-SUNDBERG, M. 2013. Colon cancer-specific cytochrome P450 2W1 converts duocarmycin analogues into potent tumor cytotoxins. *Clin Cancer Res,* 19**,** 2952-61.
TSACHAKI, M., BIRK, J., EGERT, A. & ODERMATT, A. 2015. Determination of the topology of endoplasmic reticulum membrane proteins using redox-sensitive green-fluorescence protein fusions. *Biochim Biophys Acta,* 1853**,** 1672-82.
VAN OOSTEROM, A. T., JUDSON, I., VERWEIJ, J., STROOBANTS, S., DONATO DI PAOLA, E., DIMITRIJEVIC, S., MARTENS, M., WEBB, A., SCIOT, R., VAN GLABBEKE, M., SILBERMAN, S., NIELSEN, O. S., EUROPEAN ORGANISATION FOR, R., TREATMENT OF CANCER SOFT, T. & BONE SARCOMA, G. 2001. Safety and efficacy of imatinib (STI571) in metastatic gastrointestinal stromal tumours: a phase I study. *Lancet,* 358**,** 1421-3.
WANG, K. & GUENGERICH, F. P. 2012. Bioactivation of fluorinated 2-aryl-benzothiazole antitumor molecules by human cytochrome P450s 1A1 and 2W1 and deactivation by cytochrome P450 2S1. *Chem Res Toxicol,* 25**,** 1740-51.
VASILIOU, V. & GONZALEZ, F. J. 2008. Role of CYP1B1 in glaucoma. *Annu Rev Pharmacol Toxicol,* 48**,** 333-58.
WILLIAMS, P. A., COSME, J., SRIDHAR, V., JOHNSON, E. F. & MCREE, D. E. 2000. Mammalian microsomal cytochrome P450 monooxygenase: structural adaptations for membrane binding and functional diversity. *Mol Cell,* 5**,** 121-31.

WILLIAMS, P. A., COSME, J., WARD, A., ANGOVE, H. C., MATAK VINKOVIC, D. & JHOTI, H. 2003. Crystal structure of human cytochrome P450 2C9 with bound warfarin. *Nature,* 424**,** 464-8.

WILLIAMS, P. A., COSME, J., VINKOVIC, D. M., WARD, A., ANGOVE, H. C., DAY, P. J., VONRHEIN, C., TICKLE, I. J. & JHOTI, H. 2004. Crystal structures of human cytochrome P450 3A4 bound to metyrapone and progesterone. *Science,* 305**,** 683-6.

VON HEIJNE, G. 2006. Membrane-protein topology. *Nat Rev Mol Cell Biol,* 7**,** 909-18.

WU, Z. L., SOHL, C. D., SHIMADA, T. & GUENGERICH, F. P. 2006. Recombinant enzymes overexpressed in bacteria show broad catalytic specificity of human cytochrome P450 2W1 and limited activity of human cytochrome P450 2S1. *Mol Pharmacol,* 69**,** 2007-14.

XIAO, Y. & GUENGERICH, F. P. 2012. Metabolomic analysis and identification of a role for the orphan human cytochrome P450 2W1 in selective oxidation of lysophospholipids. *J Lipid Res,* 53**,** 1610-7.

YANO, J. K., HSU, M. H., GRIFFIN, K. J., STOUT, C. D. & JOHNSON, E. F. 2005. Structures of human microsomal cytochrome P450 2A6 complexed with coumarin and methoxsalen. *Nat Struct Mol Biol,* 12**,** 822-3.

YASHIRO, K., ZHAO, X., UEHARA, M., YAMASHITA, K., NISHIJIMA, M., NISHINO, J., SAIJOH, Y., SAKAI, Y. & HAMADA, H. 2004. Regulation of retinoic acid distribution is required for proximodistal patterning and outgrowth of the developing mouse limb. *Dev Cell,* 6**,** 411-22.

YONG, K. J., GAO, C., LIM, J. S., YAN, B., YANG, H., DIMITROV, T., KAWASAKI, A., ONG, C. W., WONG, K. F., LEE, S., RAVIKUMAR, S., SRIVASTAVA, S., TIAN, X., POON, R. T., FAN, S. T., LUK, J. M., DAN, Y. Y., SALTO-TELLEZ, M., CHAI, L. & TENEN, D. G. 2013. Oncofetal gene SALL4 in aggressive hepatocellular carcinoma. *N Engl J Med,* 368**,** 2266-76.

YOSHIOKA, H., KASAI, N., IKUSHIRO, S., SHINKYO, R., KAMAKURA, M., OHTA, M., INOUYE, K. & SAKAKI, T. 2006. Enzymatic properties of human CYP2W1 expressed in Escherichia coli. *Biochem Biophys Res Commun,* 345**,** 169-74.

ZAWAIRA, A., CHING, L. Y., COULSON, L., BLACKBURN, J. & WEI, Y. C. 2011. An expanded, unified substrate recognition site map for mammalian cytochrome P450s: analysis of molecular interactions between 15 mammalian CYP450 isoforms and 868 substrates. *Curr Drug Metab,* 12**,** 684-700.

ZELDIN, D. C., FOLEY, J., GOLDSWORTHY, S. M., COOK, M. E., BOYLE, J. E., MA, J., MOOMAW, C. R., TOMER, K. B., STEENBERGEN, C. & WU, S. 1997. CYP2J subfamily cytochrome P450s in the gastrointestinal tract: expression, localization, and potential functional significance. *Mol Pharmacol,* 51**,** 931-43.

ZHANG, K., JIANG, L., HE, R., LI, B. L., JIA, Z., HUANG, R. H. & MU, Y. 2014. Prognostic value of CYP2W1 expression in patients with human hepatocellular carcinoma. *Tumour Biol,* 35**,** 7669-73.

ZHAO, Y., WAN, D., YANG, J., HAMMOCK, B. D. & ORTIZ DE MONTELLANO, P. R. 2016a. Catalytic Activities of Tumor-Specific Human Cytochrome P450 CYP2W1 Toward Endogenous Substrates. *Drug Metab Dispos,* 44**,** 771-80.

ZHAO, Y., WAN, D., YANG, J., HAMMOCK, B. D. & ORTIZ DE MONTELLANO, P. R. 2016b. Catalytic Activities of Tumor-Specific Human Cytochrome P450 CYP2W1 Towards Endogenous Substrates. *Drug Metab Dispos*.